Succinate Metabolism by

Azorhizobium caulinodans ORS 571

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by

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CONTENTS

		Page
Acknowledgements		1
Abstract		2
CHAPTER 1	Introduction	
1.1	Historical background.	3
1.2	The nitrogenase system.	4
1.3	The family Rhizobiaceae.	8
	1.3.1 The *Rhizobium*-legume symbiosis.	9
	1.3.2 Stem nodulating rhizobia.	13
1.4	Energy conservation in rhizobia.	17
	1.4.1 Cytochrome content of rhizobia.	20
	1.4.2 Molar growth yields of rhizobia.	21
	1.4.3 The energy costs of nitrogen fixation in rhizobia.	24
1.5	The effect of oxygen on diazotrophs.	26
1.6	Carbon nutrition in rhizobia.	28
	1.6.1 Free-living rhizobia.	28
	1.6.2 Bacteroid carbon metabolism.	31
1.7	Aims and objectives	34

Page

CHAPTER 2 Materials and Methods

2.1 Organism. 35

2.2 Growth medium. 35

2.2.1 Tryptone-yeast extract agar, nutrient agar, minimal-salts agar. 35
2.2.2 Batch culture. 36
2.2.3 Continuous culture. 36

2.3 Growth conditions. 37

2.3.1 Batch culture. 37
2.3.2 Continuous culture. 38
2.3.3 Dissolved oxygen control. 39
2.3.4 Fermenter inoculation. 39
2.3.5 Sampling methods. 40
2.3.6 Fermenter contamination check. 40

2.4 Measurement of fermentation parameters. 41

2.4.1 Measurment of oxygen, carbon dioxide and nitrogen in the fermenter gas streams. 41
2.4.2 Gas flow rate. 41
2.4.3 Inlet gas temperatures. 41
2.4.4 Inlet and outlet gas pressure. 41
2.4.5 Liquid flow rates. 42
2.4.6 Culture volume. 42
2.4.7 Culture density. 42
2.4.8 Culture absorbence. 43
2.4.9 Total organic carbon analysis. 43
2.4.10 Elemental CHPNO analysis. 43
2.4.11 Determination of molar growth yields. 44
2.4.12 Analysis of culture supernatants. 44

Page

2.5 *In vitro* analysis. 45

2.5.1 Preparation of washed and broken cell suspensions. 45
2.5.2 Polarographic measurement of bacterial respiration rates. 45
2.5.3 Measurement of bacterial cytochrome content. 46
2.5.4 Measurement of dicarboxylate uptake and metabolism. 47
2.5.5 Measurement of dicarboxylate accumulation. 48
2.5.6 Polyacrylamide gel electrophoresis of bacterial proteins. 49
2.5.7 Preparation of periplasmic shock fluid. 50
2.5.8 Enzyme assays. 51
2.5.9 Measurement of poly-ß-hydroxybutyrate content of cells. 53
2.5.10 Presentation of results. 53

2.6 Chemicals. 54

2.6.1 Chemical abbreviations. 54

CHAPTER 3 Growth energetics of ***Azorhizobium caulinodans***

3.1 Introduction. 55

3.2 Results. 58

3.2.1 Growth substrates. 58
3.2.2 Cellular pleomorphy. 60
3.2.3 The effect of growth conditions on respiration rates of *A. caulinodans*. 63

Page

3.2.4 The effect of growth conditions on the cytochrome content of *A. caulinodans*. 65

3.2.5 The effect of inhibitors on respiratory activity. 68

3.2.6 Determination of biomass yields on succinate, oxygen and carbon dioxide during succinate-limited growth. 73

3.2.7 The effect of nutrient limitation and growth rate on enzyme activity. 84

3.3 Discussion. 88

CHAPTER 4 The effect of succinate on the growth of *Azorhizobium caulinodans*

4.1 Introduction. 93

4.2 Results. 95

4.2.1 The effect of culture pH and potassium ion concentration on the growth of *Azorhizobium caulinodans*. 95

4.3 Discussion. 102

Page

CHAPTER 5 Nitrogen-fixing growth of *Azorhizobium caulinodans* in continuous culture

5.1 Introduction. 105

5.2 Results. 107

5.2.1 Development of a system for controlling the DOT of a chemostat culture by gas blending. 107
5.2.2 Molar growth yields of *A. caulinodans* during growth under nitrogen-fixing conditions and increasing DOT. 111
5.2.3 Cytochrome content of *A. caulinodans* during growth under nitrogen-fixing conditions and increasing DOT. 116

5.3 Discussion. 116

CHAPTER 6 C_4-dicarboxylate incorporation and metabolism in *Azorhizobium caulinodans*

6.1 Introduction. 123

6.2 Results. 126

6.2.1 [^{14}C]succinate incorporation and disappearance. 126
6.2.2 The effect of metabolic inhibitors on the rate of [^{14}C]succinate incorporation. 134

Page

6.2.3 Incorporation and metabolism of [^{14}C]maleate in *A. caulinodans*. 142

6.2.4 Transport of [^{14}C]succinate and [^{14}C]maleate. 154

6.2.5 Regulation of succinate metabolism. 158

6.3 Discussion. 164

CHAPTER 7 General discussion 167

Appendix 1

Steady-state fermentation calculation. 172

Appendix 2

Calculation of theoretical molar growth yields for succinate-limited *A. caulinodans*. 175

Appendix 3

Calculation of undissociated succinic acid concentration. 176

References 181

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ABSTRACT

Azorhizobium caulinodans ORS 571 was grown in continuous culture (30°C pH 6.8) over a range of dilution rates with ammonia as the sole nitrogen source and either succinate, oxygen or sulphate as the growth-limiting nutrient.

Molar growth yields of succinate-limited cultures indicated a P/O quotient of 2 (Y^{max}succ and $Y^{max}O_2$; 42.4 and 27.7 g cell dry wt mol^{-1} respectively). Molar growth yields of oxygen-limited and sulphate-limited cultures were lower and varied inversely with the steady-state concentration of unmetabolised succinate and K^+. The deleterious effect of succinic acid probably reflects its ability to diffuse into the cell and act as an uncoupling agent.

A. caulinodans synthesised a membrane-bound respiratory chain which contained *b*- and *c*-type cytochromes plus cytochrome oxidases aa_3 and *o*-type (probably *co*). *O*-type cytochrome oxidases were partially or completely repressed by excess oxygen, whereas cytochrome oxidase aa_3 was induced by excess oxygen.

Nitrogen-fixing cultures (DOT $\geq$1%) showed decreased levels of cytochrome *c* and increased levels of cytochrome *b*. Molar growth yields were lower than those of cells assimilating ammonia, and decreased with increasing DOT. The calculated ATP/N_2 molar ratio was 17 (assuming a P/O quotient of 2).

Washed cells from succinate-limited cultures incorporated [2,3-^{14}C]succinate at a fast initial rate followed by a slower rate of metabolism (57 nmol min^{-1} [mg cell dry wt]$^{-1}$) which remained constant for at least 30 min, and reflected succinate metabolism rather than transport. [2,3-^{14}C]maleate was metabolised only very slowly and was therefore a better substrate for measuring dicarboxylate transport, which occurred at a minimum rate of 144 nmol min^{-1} [mg cell dry wt]$^{-1}$).

[^{14}C]succinate incorporation was inhibited by unlabelled fumarate, malate and maleate, abolished by the uncoupling agent FCCP and exhibited an accumulation ratio of approximately 20. Dicarboxylate transport/ incorporation was accompanied by net alkalisation of the culture medium, and partially repressed by excess succinate or a product of its metabolism and induced by succinate, fumarate and malate. These results indicate that *A. caulinodans* actively transported dicarboxylates using a common proton symport system.

CHAPTER 1

General Introduction

1.1 Historical background

Biological nitrogen fixation is the major route by which gaseous nitrogen is introduced into the ecosystem, and accounts for approximately 2 x 10^8 tonnes N per annum (Eady *et al.*, 1987). The ability to carry out this process is confined to certain prokaryotic organisms (see list in Postgate, 1982). Some of these organisms are free-living e.g. *Anabena, Nostoc, Rhodospirillum, Klebsiella, Azotobacter* and *Clostridium*, others occur in symbiotic partnership with eukaryotic organisms e.g. *Frankia* and *Rhizobium*. Symbiotic associations are generally with plants but include some animals such as certain termites (Prestwhich and Bentley, 1981).

Rhizobia form a symbiotic association with members of the Leguminosae, a large and important group in the plant kingdom which includes crop plants such as soybean, alfalfa, pea and clover.

The understanding of plant physiology by the 1880's had reached a general acceptance that plants obtained their carbon from the atmosphere and all other essential elements from the soil. It was not until 1888 that the research of Hellriegel and Willfarth (1888) convincingly led to the conclusion that leguminous plants were the exception to the rule in their ability to use atmospheric nitrogen and that this ability correlated strongly with the presence of root nodules.

The first description of root nodules was given in 1679 by Malpighi, who concluded that they were simply subterranean insect galls. After extensive microscopical examination Woronin (1886) concluded that they contained bacteria. Convincing support for their

microbial initiation was obtained when Frank (1879) showed that nodules did not form on plants cultured in sterilized soil. Schindler (1885) was the first to describe root nodules as part of a symbiotic association, but it took a further eight years before Nobbe and Hiltner (1893) showed that nitrogen assimilation was related to the bacteroids in these root nodules. From that time on the conclusions of Hellriegel and Willfarth were generally accepted. However, a fundamental problem still remained *viz* neither the cultured bacteria isolated from root nodules nor even the excised nodules themselves, ever showed even the slightest indication that they could fix nitrogen. As a result, doubts still existed well into the twentieth century regarding the nitrogen-fixing ability of bacteroids (see Beyerinck, 1918).

1.2 The nitrogenase system

Nitrogenase is the enzyme system that catalyses the reduction of nitrogen gas to ammonia. The conventional system present in all nitrogenase-containing organisms, consists of two proteins. The first protein, a molybdenum- and iron-containing tetramer (M_r 245,000) called dinitrogenase-1 (formerly molybdoferredoxin) (Bishop *et al.*, 1988) is made up of two pairs of dissimilar subunits ($\alpha_2\beta_2$), each with a M_r of about 61,000 (Swisher *et al.*, 1977). Active dinitrogenase-1 contains four [4Fe-4S] clusters and two molecules of FeMo-cofactor (Burgess, 1984). The second protein, dinitrogenase reductase-1 (formerly azoferredoxin) is a dimer (M_r 60,500) consisting of two identical subunits each with a M_r of about 30,000 (Swisher *et al.*, 1975). Bridging the two subunits is a single [4Fe-4S] cluster.

The nitrogenase system described above (nitrogenase-1), was considered for many years to be the only system involved in nitrogen

fixation by the many different diazotrophs studied. However, two alternative systems, nitrogenase-2 and nitrogenase-3, have recently been reported for *Azotobacter vinelandii* (Robson, 1986; Chisnell *et al.*, 1988). Nitrogenase-2 replaces the molydenum in dinitrogenase-1 with vanadium and is expressed under conditions of Mo starvation, and nitrogenase-3 does not appear to contain either Mo or V. The expression of the three nitrogenases is regulated by the relative levels of molybdenum and vanadium.

The amino acid sequence of the dinitrogenase-1 protein of *Clostridium pasteurianum*, *Azotobacter vinelandii* and *Klebsiella pneumoniae* show great homology (Tanaka *et al.*, 1977; Sundaresan and Ausbel, 1981; Hausinger and Howard, 1982). The primary structure of both components appears to be highly conserved during evolution, possibly as a consequence of the special properties of the enzyme system (Eady and Smith, 1979; see Postgate, 1988).

As for chemical nitrogen fixation *via* the Haber process, the biological version *via* nitrogenase also requires high energy input to initiate the reaction, and so during electron transfer to nitrogenase ATP is hydrolysed. The amount of ATP required is variable however. *In vitro* studies on cell-free nitrogenases have suggested a value of between 12 and 16 mol ATP per mol of dinitrogen fixed. A theoretical minimum of six electrons and six protons are required to reduce one mol of dinitrogen. Nitrogen fixation is linked to proton reduction and consequently hydrogen production (Robson and Postgate, 1980). The amount of hydrogen produced can vary but a minimal stoichiometry of 1 mol hydrogen evolved per mol of dinitrogen fixed is generally accepted (Rivera-Ortiz and Burris, 1975; Burgess *et al.*, 1981; Simpson and Burris, 1984), leading to an overall reaction of:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$$

In addition to nitrogen and proton reduction, nitrogenase is able to reduce a number of alternative substrates including acetylene, cyanide, azide, nitrous oxide and the non-catalytic reduction of oxygen (Thorneley *et al.*, 1988).

The flow of electrons through the nitrogenase system is from either ferredoxin or flavodoxin to the dinitrogenase-reductase-1 component (Fe protein), which then reduces the dinitrogenase-1 component (MoFe protein) (Hageman and Burris 1980; see Haaker and Klugkist, 1987). The dinitrogenase-1 protein contains the iron-molybdenum cofactor (FeMo-co) which is the probable site of substrate reduction (Hageman and Burris, 1980; Smith *et al.*, 1984). Thornely and Lowe (1983, 1984; see Haaker and Klugist, 1987) proposed that the dinitrogenase-reductase-1 received one electron from a low potential donor and bound two molecules of MgATP. The reduced Fe-MgATP complex then bound to the dinitrogenase-1 component, and electron transfer through the FeMo binding site to nitrogen, protons and intermediates was coupled to ATP hydrolysis. After electron transfer the two nitrogenase proteins dissociated and the cycle started again.

The *in vivo* biosynthesis and activity of the nitrogenase system is organised on a gene cluster (*nif*) comprising at least 21 genes, the expression of which is regulated by the *nifAL* operon. The organisation of *nif* and the various gene product interactions are complex, and a full account is beyond the scope of this thesis. For a comprehensive description of *nif* organization in relation to nitrogenase activity see Dixon, (1988); Merrick (1988*a*,*b*), and Postgate and Eady, (1988).

The genetic organization of nitrogenase in *A. caulinodans* is still under study. However, *nifE*, and the *nifHDK* genes are reported homologous with the same genes of *Klebsiella pneumoniae* (Donald *et al.*, 1986; Norel and Elmerich, 1987).

The expression of nitrogenase activity is strongly affected by the intracellular concentrations of ammonia and oxygen. In all free-

living diazotrophs studied so far, synthesis of nitrogenase is repressed after the addition of ammonium to the culture medium (Daesch and Mortenson, 1968; Tubb and Postgate, 1973; Kurz and LaRue 1975; Zumft and Castillo, 1978). The precise mechanism is unresolved. However, regulation is believed to occur through the interaction of the genes controlling glutamine synthetase activity (*gln* genes) and the *nifAL* operon (Daniel and Zumft, 1983). The *nifA* and *nifL* gene products in turn activate and repress all of the other *nif* genes (see Merrick, 1988*a*), the repressor *nifL* product is inactivated through combination with a regulatory metabolite (possibly guanosine-5′diphosphate 3′-diphosphate; ppGpp) when nitrogenase is active. Following the addition of ammonium the concentration of regulatory metabolite decreases and the *nifL* product represses *nif* transcription. An additional "rapid switch-off" mechanism has been reported for aerobic diazotrophs (Eady, 1981; Rodionov *et al.*, 1986) where inhibition of nitrogenase is related to the stoppage of ATP production for nitrogenase, caused by a collapse of the $\Delta \Psi$.

A very low dissolved oxygen concentration is also required for derepression and function of the *nif* genes, and for the activity of nitrogenase (Bergersen and Turner, 1975, 1976; Keister, 1975; Bergersen *et al.*, 1976). For example, when *Klebsiella pneumoniae* was cultured at approximately 6 μM O_2 nitrogenase was inactivated and transcription from all the *nif* operons except for *nifLA* (the regulatory operon)was inhibited (Dixon *et al.*, 1980; Hill *et al.*, 1981; Merrick *et al.*, 1982; Cannon *et al.*, 1985). At O_2 concentrations above 6 μM, expression from the *nifLA* promoter was also inhibited. The exact mechanism whereby O_2 regulates the expression of the *nif* genes is not yet fully understood. The dinitrogenase-reductase-1 (Fe protein) of nitrogenase is extremely sensitive to oxygen (Dalton and Postgate, 1969), and inhibited at dissolved oxygen concentrations above 2 μM.

In nitrogen-fixing photosynthetic bacteria, high levels of oxygen cause the Fe-protein to become inactivated through the

attachment of modifying groups consisting of phosphate, pentoses and an adenine-like moiety (Ludden and Burris, 1978). An oxygen-sensitive, membrane-bound, activating enzyme has been shown to remove the modifying groups and allow nitrogen fixation to resume once the oxygen concentration has diminished below a critical threshold level (Hallenbeck *et al.*, 1982).

1.3 The family Rhizobiaceae

The family Rhizobiaceae is subdivided into four genera: *Rhizobium*, *Bradyrhizobium*, *Agrobacterium* and *Phyllobacterium*. All species with the exception of *Agrobacterium radiobacter*, form associations with plants.

Nodules are produced on roots of leguminous species by strains of rhizobia, and lesions on leaves of certain plants by strains of phyllobacteria. Gall hypertrophies are produced on roots and stems on a wide range of plant species by strains of *Agrobacterium*.

The bacteria are typically Gram-negative, aerobic, rod-shaped and do not form endospores. Many species produce large quantities of exopolysaccharides especially when cultured under carbon-excess growth conditions (Zevenhuizen, 1971; Zevenhuizen, 1984; Hollingsworth *et al.*, 1985). Polysaccharide production has also been observed for rhizobia growing in the rhizosphere and associated with the infection process (Sanders *et al.*, 1981; Borthakur *et al.*, 1985). Traditionally, the classification of rhizobia was based upon the specificity of the host-symbiont relationship. In addition a gross division has been made between the so called slow-growing, alkali-producing, and the so called fast-growing, acid-producing strains, (Norris, 1964). Slow growers include *Bradyrhizobium japonicum* (formerly *Rhizobium japonicum*) and *R. lupini* which nodulate soybeans and lupins respectively, and fast growing species include

R. meliloti, *R. trifolii*, *R. leguminosarum* and *R. phaseoli* which nodulate alfalfa, clover, pea and field beans respectively. The designation alkali- or acid-producing is of little value since such phenomena are a function of the media in which growth occurs. But the division between fast- and slow-growing strains does have a physiological and genetic basis (Martinez de Drets and Arias, 1972; Dudman, 1976; Duncan, 1979; Beringer *et al.*, 1980; Brewin *et al.*, 1980), and led to the division of the genus *Rhizobium* into two distinct genera. The new genus, *Bradyrhizobium* (Jordan, 1982) was created principally as a result of the DNA/DNA hybridization studies of Hollis *et al.* 1981.

A miscellaneous group is formed by the so called cowpea-rhizobia. These rhizobia are essentially slow growers but differ from *B. japonicum* in their ecological distribution and abilities to nodulate a broad range of legume species (Ahamad *et al.*, 1981; Allen and Allen, 1981).

It has been suggested that *Rhizobium* species are chromosomally identical and differ only in plasmid-located genes involved in the nodulation process (Brewin *et al.*, 1980).

1.3.1 The *Rhizobium*-legume symbiosis.

The legume root (or stem) nodule is the result of a complex series of plant-microbe interactions (see Halverson and Stacey, 1986), which eventually lead to an intracellular symbiotic association, where the host plant provides both a suitable environment and all the nutritional requirements for the bacteroids to fix atmospheric nitrogen. However, the immediate benefits for the rhizobia are not obvious. Indeed the infection of legumes has been considered as a beneficial plant disease (Vance, 1983), since doubt exists as to whether differentiated bacteroids can regenerate into free-living

bacterial cells, re-enter the rhizosphere and colonise other legumes.

The processes leading to nodule formation have been extensively reviewed by Halverson and Stacey (1986). Cell colonization is normally accomplished *via* an infection thread (Bauer, 1981) originating from the point of entry, usually a root hair or an epidermal fissure. The sequence of events leading to production of infection threads is not fully understood, the selective interaction of host plant lectins with compatible *Rhizobium* cell surface polysaccharides has been proposed (see Albersheim and Anderson-Prouty, 1975), and more recently host plant flavonoids have been suggested as inducers of the nodulation genes in *Rhizobium* (Redmond *et al.*, 1986; Firmin *et al.*, 1986). In or during release from the thread, the bacteria differentiate into bacteroids, and lose their cell wall structure (Sutton *et al.*, 1981) (Fig 1.1). All Fix^+ and some Fix^- strains are surrounded by a host-derived membrane, termed the peribacteroid membrane (Newcomb, 1981; Robertson *et al.*, 1984), the structure and biogenesis of which has recently been reviewed by Mellor and Werner (1987).

Enclosure of the bacteroids by a plant-derived membrane has a number of important consequences. Significant problems could arise concerning the entry of inorganic nutrients and carbon compounds into the bacteroid, and for the exit of fixed nitrogen, principally due to potential controls on the transport of materials between the plant and bacteroid by two membrane systems rather than one.

The activities ocurring in the peribacteroid space (the space between the bacteroid and peribacteroid membrane) are not fully understood. Hydrolytic, proteolytic and glycolytic enzyme activities have all been detected (Mellor *et al.*, 1984; Fortin *et al.*, 1985). The origins of these enzymes are unclear but they are thought to be the bacteroid (Robertson *et al.*, 1985).

Probably the most significant role of the peribacteroid membrane is to regulate the transport of solutes. The latter include

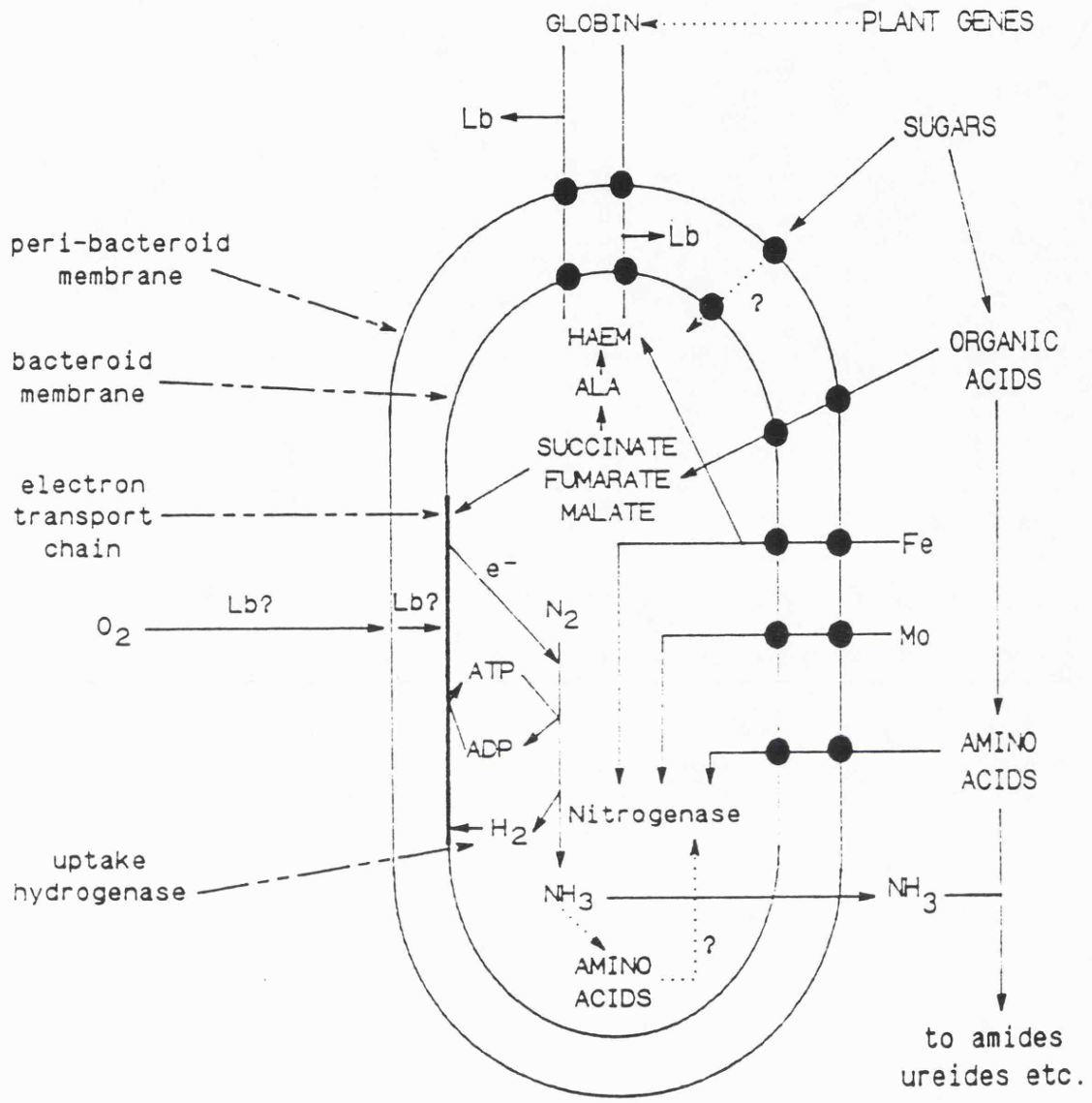


Figure 1.1. Schematic diagram of the metabolic exchanges between plant and bacterial cells in a nitrogen-fixing nodule. Transport systems, ● ; potential pathways, ·····; leghaemoglobin, Lb; 5- amino-levulinic acid, ALA. (Adapted from Dilworth and Glenn, 1984).

amino acids, organic acids, possibly sugars, trace elements (especially iron and molybdenum) and vitamins, all of which enter the bacteroid (Sutton *et al.*, 1981; Verma and Long, 1983), and ammonia which leaves. The exported ammonia is assimilated in the plant cytosol by glutamine synthetase, the high activity of which is sufficient to maintain a concentration gradient for the continued diffusion of ammonia from the bacteroid to the plant (see Sprent, 1984).

The electron supply for nitrogenase is derived ultimately from the carbon compounds imported into the bacteroid (see section 1.4.2). Dicarboxylic acid transport mutants (Dct^-) are able to nodulate but unable to fix atmospheric nitrogen (Fix^-) (Ronson *et al.*, 1981), as also are succinate dehydrogenase mutants (Sdh^-) (Finan *et al.*, 1983), thus suggesting a specific role for succinate, fumarate or malate in nitrogen fixation. ATP generation in bacteroids (and free-living cells) is primarily through oxidative phosphorylation, and so requires the presence of oxygen. This requirement poses two problems for the symbiosis. Firstly, nitrogenase is inactivated by oxygen concentrations above 6μM, and its synthesis is also repressed (Hill *et al.*, 1981). Secondly, oxygen is only freely available at the legume-cell boundary, and the high density bacteroid population in the nodule may result in poor oxygen transfer towards the centre of the nodule. Bacteroids in this latter region may receive insufficient oxygen and so become oxygen-limited for nitrogen fixation (see Bergersen, 1982). A balance must therefore be achieved allowing maximum nitrogen-fixation and minimum nitrogenase inactivation. This problem has been solved in part by production of leghaemoglobin (see Appelby, 1984).

Leghaemoglobin serves two major functions. Firstly, the high concentration of this oxygen-binding protein facilitates the rapid diffusion of oxygen into the cell (Appelby *et al.*, 1975). Secondly, it ensures that the steady-state concentration of free oxygen is very low and hence unlikely to inactivate nitrogenase. The precise

location of leghaemoglobin remains controversial, although, the most compelling evidence suggests that it is located in the peribacteroid space (Goodchild, 1977).

In addition to leghaemoglobin, the oxygen status of the nodule may be controlled by a diffusion barrier comprised of a band of cortical cells that can vary their internal volume and so regulate the diffusion of gas through the aqueous barrier (Witty *et al.*, 1986).

Leghaemoglobin is possibly a product of both the bacteroid and host genome. The globin proteins are coded for by the plant and the protohaem synthesis appears to be bacterial. Nevertheless, the evidence that protohaem is uniquely a bacteroid product is uncertain. However, free haem is not found in the plant cytosol (Nadler and Avissar, 1977; Appelby, 1984). If leghaemoglobin is truly a symbiotic product then the concept of rhizobial infection as a beneficial disease would be in doubt.

1.3.2 Stem-nodulating Rhizobia.

Nitrogen-fixing nodules are usually found on the roots of host leguminous plants. However, some legume species belonging to the genera *Neptunia* and *Aeschynomene* form nodules on the stem (Hagerup, 1928; Schaede, 1940), and those of the genus *Sesbania* form nodules on both the stem and root (Dreyfus and Dommergues, 1981). Dreyfus *et al.* (1984) reported that the tropical legume *Sesbania rostrata* was associated with two distinct strains of rhizobia. The first type, which were able to grow with atmospheric nitrogen gas as the sole nitrogen source and to nodulate both the stems and roots of *S. rostrata* (Dreyfus *et al.*, 1983; Gebhardt *et al.*, 1984), were initially designated *Rhizobium sesbania* (Dreyfus and Dommergues,

1981) but were later reclassified as *Azorhizobium caulinodans*, of which the type strain is *A. caulinodans* ORS 571 (Dreyfus *et al.*, 1988). The second type, which nodulated only the roots of *S. rostrata* and did not fix atmospheric nitrogen in culture, were classified simply as *Rhizobium* sp. ORS (Dreyfus *et al.*, 1988). Stem nodulation of *Sesbania* spp. is not restricted to *S. rostrata* but has also been observed with *S. vesicaria* and *S. drumondii* (Brown *et al.*, 1985). For a complete taxonomic description of *A. caulinodans* ORS 571, and classification of the *Azorhizobium* genus see Dreyfus *et al.* (1988).

Stem-nodulated legumes are relatively common and distributed over wide areas of tropical savanna. A common feature shared by all of this group is that they inhabit for at least part of their life-cycle waterlogged soil (Dreyfus *et al.*, 1984). Sites of stem nodulation are predetermined, in contrast to their root counterparts, and always include a dormant root primordium. Nodulation is initiated when the root primordium grows and breaks through the epidermal surface causing fissures which are then invaded by the rhizobia (see Tsien *et al.*, 1983). The subsequent stages of nodulation; *viz* host-symbiont recognition, the formation of infection threads and bacteriod differentiation are very similar to the processes already well-documented for the root.

Considerable interest has been focused on *S. rostrata* and *A. caulinodans* over the past few years. *S. rostrata* grows extremely rapidly, and has a high organic nitrogen content (Sidhu *et al.*, 1985). These characteristics and its availability to farmers in tropical areas make it an excellent green manure which is especially attractive to those who are unable to afford conventional inorganic fertilisers (Rinaudo *et al.*, 1983; Sidhu *et al.*, 1985).

The interest in *A. caulinodans* arose essentially from the ability of the organism to grow on atmospheric nitrogen *ex planta*. No other rhizobia expresses this characteristic to the extent of *A. caulinodans*, although derepression of nitrogenase has been demonstrated in a restricted number of rhizobia, principally *Bradyrhizo-*

bium japonicum and cowpea-rhizobia strains (Holsten *et al.*, 1971; Child and La Rue, 1974; Phillips, 1974).

The nitrogenase proteins of *A. caulinodans* were first characterised by Kush *et al.* (1985). Both components were purified to homogeneity; the Mo-Fe protein (dinitrogenase-1) had a M_r of 219,000, consisting of two types of subunit (M_r 56,000 and 59,000) in a mixed ($\alpha_2\beta_2$) tetramer, and the Fe-protein dimer (dinitrogenase-reductase-1) (M_r 74,000) consisted of one type of subunit (M_r 36,000). Two copies of *nifH* (*nifH1* and *nifH2* that differ by six nucleotides have been reported for *A. caulinodans* (Norel and Elmerich, 1987). The significance of these copies is unclear but they may perhaps be involved with an alternative nitrogenase as found in *Azotobacter* (Bishop *et al.*, 1988). The nitrogenase was subject to "switch-off" when ammonia was added to the nitrogen-fixing culture, which resulted from a decrease in activity of dinitrogenase-reductase-1, perhaps due to the attachment of modifying groups (see Ludden and Burris, 1978). The effect was independent of protein synthesis and was reversible. The structure and physico-chemical properties of the two components were very similar to previously characterised nitrogenases (see section 1.2).

The genetics of nitrogenase in *A. caulinodans* are still under study. However, the genetic organisation appears to be similar to that reported for other nitrogenase containing organisms (Donald *et al.*, 1986; Norel and Elmerich, 1987).

Stem and root nodules of *S. rostrata* contain leghaemoglobins which appear to differ slightly in composition following chromatographic fractional analysis (Bogusz, 1984), but are sufficiently similar to leghaemoglobin from soybean and alfalfa for antisera to cross react. Superoxide dismutase (SOD) activity was reported in both stem and root nodule bacteroids by Puppo *et al.* (1986). The enzyme was also present in free-living *A. caulinodans*. The SOD may remove $O_2^{\cdot -}$ free radicals generated from the reduction of oxygen by nitrogenase, or in the bacteroid to protect leghaemoglobin from

accidental auto-oxidation.

The ammonium assimilation pathway in free-living *A. caulinodans* was reported to consist of glutamine synthetase and glutamate synthase (Donald and Ludwig, 1984) However, only one of the two glutamine synthetase isoenzymes normally present in rhizobia (Fuchs and Keister 1980) was found (Donald and Ludwig, 1984). Glutamine synthetase was virtually undetectable in bacteroids, which was in keeping with the generally accepted idea that bacteroids "export" all of the fixed nitrogen as ammonia, and obtain their nitrogen in the form of amino acids imported from the host cytosol.

Holsters *et al.* (1985) isolated two types of *A. caulinodans* mutants which were deficient in nitrogen fixation. The first (*Nif*$^-$), was unable to fix nitrogen in either the symbiotic or free-living state, and the second (*Fix*$^-$) was able to fix nitrogen only in the free-living state but formed ineffective nodules.

The ability of rhizobia to fix nitrogen *ex planta* only occurs in microaerobic conditions. However, free-living *A. caulinodans* is considerably more tolerant to external oxygen concentrations. Dreyfus *et al.* (1983) reported good growth of *A. caulinodans* in a gaseous environment composed of 3% oxygen and 97% nitrogen, but the growth rate declined markedly at 5% oxygen and ceased when air was bubbled through the culture. Stam *et al.* (1984) reported very similar findings. Gebhardt *et al.* (1984) found that in continuous culture (D=0.10 h^{-1}), nitrogenase activity, bacterial cell density and cell nitrogen content were strongly decreased when the culture dissolved oxygen concentration exceeded 9 μM, which was similar to the value of 12 μM reported by Stam (1986). In contrast, Stouthamer *et al.* (1988) reported nitrogen-fixing growth of *A. caulinodans* at dissolved oxygen concentrations up to 65 μM. However, in the latter study the cell density was low (between 0.5-0.1 gl^{-1}, as calculated from culture absorbence readings), and it was not inconceivable that the bacteria were growing on added fixed nitrogen, principally nicotinic acid (see Ludwig, 1986).

1.4 Energy conservation in rhizobia

Respiration is characterised by the transfer of reducing equivalents (H,H^-,e^-) from an exogenous donor to an exogenous acceptor *via* a series of sequential oxidation-reduction reactions. These reactions are catalysed by membrane-bound, spatially-organised redox carriers, which collectively make up the respiratory chain. Overall, they liberate an amount of free energy which is determined by the difference between the redox potentials of the donor and acceptor couples, and therefore different types of respiration can be accompanied by widely differing energy yields (see Jones, 1985).

All known respiratory systems exhibit some evidence of branching, but the extent and physiological importance of this phenomenon is extremely variable (see White and Sinclair, 1971; Jones, 1977). Extensive branching can occur at the level of the primary dehydrogenases but also at the terminal end of the respiratory chain. In most cases the branching is limited to electron transfer from a common penultimate *b*- or *c*-type cytochrome to molecular oxygen *via* the available terminal oxidases (e.g. in *Micrococcus lysodeikticus*, *Alcaligenes eutrophus* and *Bacillus megaterium*). However, in a limited number of systems branching is more complex and appears to involve *b*- and/or *c*-type cytochromes as well as multiple cytochrome oxidases; e.g. *E. coli*, *Azotobacter vinelandii* and *Beneckea natrigens* grown under oxygen-limited conditions (Jones and Redfearn, 1967; Yates and Jones, 1974; Linton *et al.*, 1977; see Jones, 1977, 1985).

Variations in redox carrier patterns can be induced within a single species by altering the growth conditions, particularly with respect to the availability of molecular oxygen; these variations occur principally amongst the terminal cytochrome oxidases and quinones (Jones, 1977).

The major function of a branched respiratory system is almost

certainly to allow some flexibility in the exact route of electron transfer, thus enabling the cell to minimise the potentially deleterious effects of certain growth environments and to take maximum advantage of others (Jones, 1985).

Bacterial respiration, like other metabolic processes, is subject to two basic types of regulation, *viz* coarse control, which is effected *via* the repression and induction of redox carrier biosynthesis (e.g. during oxygen limitation) and fine control, which is effected *via* the inherent kinetic and thermodynamic properties of the respiratory system. In terms of fine control, dehydrogenase activities are regulated by the availability of the various reducing substrates or by the direct action of adenine nucleotides (for a description of respiratory regulation *in vivo* see Harrison, 1972; Jones, 1977).

According to the chemiosmotic hypothesis of Mitchell (1961), respiratory electron transfer can be described by the general equation :

$$DH_2 + A + zH^+_{(in)} \rightarrow zH^+_{(out)} + AH_2 + D$$

where D and A are the electron donor and receptor respectively and z is numerically equal to the $\rightarrow H^+/O$ ($\rightarrow H^+/2e^-$) quotient (g-ion H^+ per g-atom O or mole of alternative two electron acceptor reduced)$^{-1}$ (Jones, 1982).

The stoichiometry of ATP synthesis during oxidative phosphorylation is expressed as the ATP/O (P/O), ATP/$2e^-$ quotient (mole ATP synthesised or phosphate esterified) per (g-atom O or mole of alternative two electron acceptor reduced)$^{-1}$.

Similarily, ATP synthesis may be described by the equation :

$$H^+ + ADP^{3-} + HPO_4^{2-} + xH^+_{(out)} \rightarrow xH^+_{(in)} + ATP^{4-} + H_2O$$

where x is numerically equal to the $\rightarrow H^+/ATP$ ($\rightarrow H^+/P$) quotient (g-

ion H^+ per mole ATP synthesised or phosphate esterified^{-1}). The ATP/O (ATP/$2e^-$) quotient is therefore equal to the $\rightarrow H^+/O$ ($\rightarrow H^+/2e^-$) quotient divided by the $\rightarrow H^+$/ATP quotient (Jones, 1977).

In comparison with the relatively large amount of information which is available in the literature concerning the composition of bacterial respiratory chains, much less is known about the efficiencies with which they conserve energy. The main reasons for this imbalance are almost certainly related to the experimental difficulties of assaying oxidative phosphorylation in bacterial cells and respiratory membranes derived from them. Oxidative phosphorylation can be assayed by measuring either respiration-induced changes in the composition of endogenous adenine nucleotide pools (see Baak and Postma, 1971), respiration-linked proton ejection ($\rightarrow H^+/O$ quotients) (see Scholes and Mitchell, 1970) or by determining molar growth yields.

The organisation of bacterial respiratory chains into proton translocating segments is well documented. Four segments or sites are recognised (sites 0, I, II and III) however, only sites I, II and III are believed to correspond to energy conservation segments where ATP synthesis occurs. The first site (site 0) is a transhydrogenase which effects the reversible transfer of a hydride ion (H^-) between NADPH and NAD^+. The equilibrium of the reaction tends to favour NADPH formation from NADH, but nonetheless it does represent a potential site of energy conservation. Site I is comprised of NADH dehydrogenase and site II a cytochrome *b/c* complex. ATP synthesis at site III depends on the presence of cytochrome *c* linked to cytochrome oxidase aa_3 or *o*.

Sites I and II are generally constitutive, whereas site 0 and III are dependent on the variable abilities of different organisms to synthesise significant amounts of transhydrogenase and a high potential cytochrome *c* linked to cytochrome oxidase aa_3 or *o*. In organisms which lack a cytochrome c of this type , respiration is terminated by cytochrome oxidases aa_3, *o* or *d* immediately after the

Q-*b* region (Jones *et al.*, 1977).

1.4.1 Cytochrome content of rhizobia.

The absorption spectrum of free-living rhizobia shows the presence of cytochromes *c*, *b* and cytochrome oxidases aa_3, *o*, *co* and *d*, suggesting significant branching at the level of the terminal oxidase (Appelby, 1969, de Hollander and Stouthamer, 1980). Ratcliffe *et al.* (1983) reported a cytochrome pattern of (Q),*b*,*c*,aa_3,*o* for *R. leguminosarum* following glucose-limited growth in continuous culture under highly aerobic conditions (206 μM O_2). This pattern changed to (Q),*b*,*(c?)*,aa_3,*o*,*d* during oxygen-limited conditions. The concentration of cytochrome c_{550} decreased in parallel with decreasing oxygen concentration, whereas, the concentrations of cytochromes c_{555} and b_{560} increased with decreasing dissolved oxygen concentration. Similar findings were reported by de Hollander (1981) for *R. trifolii* following changes in culture oxygen concentration, which suggested that the majority of electrons were routed through the (Q),*b*,*d* pathway as already described for oxygen-limited *Escherichia coli*.

Stam *et al.* (1983) reported a cytochrome profile of (Q),*b*,*c*,aa_3,*o* for succinate-limited *A. caulinodans* grown under highly aerobic conditions. However, under oxygen-limited conditions the concentration of cytochrome oxidase aa_3 decreased markedly (in contrast to *R. leguminosarum* (Ratcliffe *et al.*, 1983), but the cytochrome *c* concentration remained the same. The presence of cytochrome oxidase *d* during oxygen-limited growth was reported by Stam *et al.* (1983) but not substantiated.

As with rhizobia cultured under non nitrogen-fixing conditions, bacteroids contain cytochrome *c* and cytochrome *b*, but exclusively contain cytochrome c_{552} and the haemprotein P-450 (Appelby,

1969, 1984). However, they are reported to lack cytochrome oxidases aa_3, *d* and *o* found in cultured cells (Appelby, 1969), although O'Brian and Maier (1983) reported cytochrome oxidase *o* in hydrogenase positive *B. japonicum* bacteroids (see section 1.4.3).

1.4.2 Molar growth yields of rhizobia.

The importance of assessing the efficiency of bacterial energy conservation by the measurement of molar growth yields (g cell dry wt mol^{-1} substrate/product) has been realised for many years (see Stouthamer 1979). Initially measurements were confined to anaerobic cultures but later extended to aerobic organisms (where oxidative phosphorylation replaces substrate phosphorylation as the major energy-conserving process). During anaerobic growth, cell yields are most usefully expressed in terms of the amount of carbon substrate which is fermented to provide a source of energy, e.g. Ycarbon substrate (g cells per mol carbon substrate consumed). This value reflects both the efficiency of energy conservation *via* substrate-level phosphorylation and subseqently the efficiency with which this energy is used to drive biosynthesis and other energy-dependent cellular reactions. Since the substrate-level phosphorylation reactions of bacterial cells are well documented it is relatively easy to calculate the Y_{ATP} (g cells per mol ATP) from the Ycarbon substrate. In contrast, for aerobic bacteria the relationship of oxidative phosphorylation to the efficiency of growth is rather more complicated. The aerobic growth yields are most usefully expressed in terms of the amount of oxygen consumed during *in vivo* respiration (Yo_2; g cells per mol O_2) which is equal to the product of *N* (the overall efficiency of aerobic energy conservation i.e. oxidative phosphorylation plus a small contribution from substrate-level

phosphorylation) and Y_{ATP} (see Jones *et al.*, 1977);

$$Yo_2 = Y_{ATP} \cdot N$$

The overall efficiency of aerobic energy conservation can also be obtained from the the Ycarbon substrate;

$$\text{Ycarbon substrate dissimilated} = Y_{ATP} \cdot N \cdot Z$$

where Z is the amount of oxygen consumed during the complete oxidation of the carbon substrate (mole O_2 per mole substrate^{-1}). Thus, in order to calculate the efficiency of oxidative phosphorylation from Yo_2 or Ycarbon substrate, it is necessary to know the Y_{ATP} and also to make a small correction for substrate-level phosphorylation.

Much of the early work in this field took no account of energy required for cell maintenance and growth, and was carried out in batch cultures of changing nutritional status. However the use of continuous culture techniques has enabled growth yields to be corrected for the influence of maintenance (Pirt, 1975). The efficiency of aerobic growth under energy-limited continuous culture conditions can be described by the equation;

$$\frac{1}{Yo_2} = \frac{1}{Y^{max}o_2} + \frac{Mo_2}{\mu}$$

where Y^{max} is the maximum molar growth yield (g cells per mol O_2), Mo_2 is the maintenance respiration rate and μ the specific growth rate (h^{-1}; equivalent to the dilution rate D of a continuous culture). A similar relationship exists for the Ycarbon substrate. However, by definition this relationship only holds true when the rate of biomass formation is strictly dependent on the rate of substrate consumption (see Tempest, 1978).

Where *in vitro* studies of respiratory chain conservation have

been supplemented by *in vivo* determination of maximum molar growth yields of energy-limited continuous cultures, the results generally indicate that organisms which route the majority of their terminal electron flow *via* a cytochrome *c* → aa_3/o system exhibit 50% higher molar growth yields than organisms which lack cytochrome *c* (Jones *et al.*, 1977).

Few reports have been published detailing the molar growth yields of rhizobia; De Hollander and Stouthamer (1979) determined the molar growth yields in mannitol/asparagine-limited cultures of *R. trifolii*, and reported a Y^{max}mannitol/asparagine of 68 g cell dry wt mol^{-1} and a calculated Y_{ATP} of 8 g cell dry wt mol^{-1}. The significance of this study is unclear since asparagine was used as both carbon and nitrogen source. Stam *et al.* (1983) reported a Ymannitol of 33 g cell dry wt mol^{-1} for *R. leguminosarum* grown under mannitol limitation in continuous culture. Ratcliffe *et al.* (1983) measured the molar growth yields for *R.leguminosarum* in glucose-limited culture, and reported Y^{max}glucose and Y^{max}oxygen values of 80.3 and 25.6 g cell dry wt mol^{-1} respectively. These molar growth yields were considered low compared to other glucose-limited organisms which also contained cytochrome *c* (Jones *et al.*, 1977), and were closer to values obtained from bacterial systems which lacked this cytochrome. The observed efficiency of *R. leguminosarum* was similar to a bacterium with a P/O quotient of 1 although $\rightarrow H^+/O$ measurements suggested a potential P/O quotient of 3. Stam *et al.* (1984) reported $Y_{succinate}$ and Yo_2 values of 43.2 and 27.0 respectively, for *A. caulinodans* grown in succinate-limited continuous culture (D = $0.10h^{-1}$). These values were also lower than expected for a cytochrome *c* containing strain, and $\rightarrow H^+/O$ measurments suggested a P/O quotient of between 2 and 3. The calculated Y^{max}oxygen of de Hollander and Stouthamer (1979) of approximately 33.3 g cell dry wt mol^{-1} for *R. trifolli* was also low, suggesting a poor coupling between respiration and energy generation.

The number of sites of proton translocation in bacteroids is

unknown. However, an $\rightarrow H^+/O$ quotient of 6.4 for the oxidation of endogenous substrates was reported for isolated bacteroids of *B. japonicum* (Ratcliffe *et al.*, 1980). The presence of cytochrome c_{550} was also reported suggesting 3 potential sites of proton translocation.

1.4.3 The energy costs of nitrogen fixation in rhizobia.

The energy costs of nitrogen fixation are very hard to determine. However, by comparing the molar growth yields of ammonia-assimilating and nitrogen-fixing chemostat cultures the exact cost in terms of ATP can be calculated for anaerobic and estimated for aerobic systems (due to the reasons described above). Anaerobic nitrogen-fixing organisms have yielded experimental ATP : N_2 ratios of between 20 and 30 for *Clostridium pasteuranium* and *Klebsiella pneumoniae* respectively (Daesch and Mortensen, 1968; Hill, 1976). Unfortunately, very little data is available for aerobic systems although Stam *et al.* (1983) reported a decrease in the Ymannitol from 33 to 15 g cell dry wt mol^{-1} following the derepression of nitrogenase activity. ATP : N_2 ratios of between 45 and 50 have been derived for *A. caulinodans* grown under succinate limitation (Stam *et al.*, 1984; de Vries *et al.*, 1984). However, large differences may exist between *Rhizobium* cells growing in culture and *Rhizobium* bacteroids fixing nitrogen within root nodules.

Nitrogen fixation is a highly energy-consuming process partly because hydrogen is produced during the reduction of nitrogen to ammonia. Some nitrogen-fixing organisms recycle the evolved hydrogen *via* an uptake hydrogenase, regaining part of the energy initially consumed.

The hydrogenase system has been found in bacteroids and free-living nitrogen-fixing cells of *Bradyrhizobium japonicum* cowpea-

rhizobia and *A. caulinodans* (Arp and Burris, 1979, Harker *et al.*, 1984, Stam *et al.*, 1984). The enzyme is an Ni-Fe protein that oxidises hydrogen and donates electrons through ubiquinone to cytochromes *b*, *c* and cytochrome oxidase *o* (O'Brian and Maier, 1983).

The hydrogenase is capable of performing three functions in the cell; electron transport can be coupled to ATP synthesis (as briefly described above), uncoupled electron transport may consume intracellular oxygen which in turn could inactivate nitrogenase, and the hydrogenase may remove hydrogen that may also inhibit nitrogenase activity (Dixon, 1972).

The yield of ATP generated *via* hydrogenase activity is very hard to determine. However, reasonable estimates have been made by comparing the molar growth yields of nitrogen-fixing cells grown in continuous culture under different hydrogen/nitrogen ratios with hydrogenase positive (Hup^+) and hydrogenase negative (Hup^-) strains. Stam *et al.* (1984) clearly showed that hydrogenase activity in nitrogen-fixing *A. caulinodans* increased the Ysuccinate from 23.6 to 25.1 g cell dry wt mol^{-1} with an hydrogen/nitrogen quotient of 10.

Considerable interest has been focused on Hup^+ rhizobia strains as commercial inoculants with respect to their reported ability to increase the dry matter and nitrogen content of leguminous crops (see Eisbrenner and Evans, 1983), although doubt over the extent and significance of the yield increases has also been expressed (Nelson and Child, 1981; Nelson, 1983; Cunningham *et al.*, 1985).

However, the energy loss during hydrogen evolution can never be totally compensated for by hydrogenase activity. Ratcliffe *et al.* (1980) calculated only 53% efficiency of energy conservation with 3 sites of proton translocation following hydrogen oxidation. Under oxygen-limited conditions (as may occur in nitrogen-fixing bacteroids), the process is energetically unfavorable as it yields less energy than the oxidation of endogenous substrates (Stam, 1986).

1.5 The effect of oxygen on diazotrophs

In rhizobia, Bergersen (1977) observed nitrogenase activity with cowpea-*Rhizobium* at dissolved oxygen concentrations of 1 μM but this activity ceased above 30 μM. De Hollander (1981) and Stam *et al.* (1983) also reported similar results for cultures of *R. trifolii* and *R. leguminosarum* respectively. However, Stam *et al.* (1984) and Gebhardt *et al.* (1984) reported nitrogen-fixing growth of *A. caulinodans* in continuous culture at dissolved oxygen concentrations of between 30 μM and 40 μM, considerably higher than for other rhizobia. The reasons for this high tolerance were unclear.

As a result of oxygen inactivation, protection of nitrogenase is a widespread phenomenon amongst free-living aerobic diazotrophs. The protective mechanisms fall into three principle categories *viz* conformational protection of the dinitrogenase-reductase protein, compartmentalisation of the nitrogenase (spatial separation) and respiratory protection.

Studies on the influence of oxygen on nitrogen fixation in chemostat cultures of *Azotobacter chroococcum* led to the proposal that in addition to active protection by respiration, oxygen stress could cause nitrogenase *in vivo* to assume a conformation in which it was protected from oxygen (switch-off), and activity could be restored rapidly when the oxygen concentration was lowered (switch-on) without protein synthesis *de novo*. This conformational protection results from an association between the nitrogenase and another Fe-S protein. Interestingly, the vanadium nitrogenase of *A. chroococcum* lacks the conformational protection available to its molybdenum containing counterpart (Bishop *et al.*, 1988).

Compartmentalisation is where the nitrogenase system is spatially separated from areas of high oxygen concentration. An example of this type of protection is the formation of heterocysts in certain cyanobacteria spp. These specialised cells are thick walled to

limit diffusion of oxygen into the cell. Although the heterocysts are photosynthetic, they lack photosystem II (whilst retaining photosystem I) and so generate ATP *via* cyclic photophosphorylation. The heterocysts do not fix CO_2 or generate large quantities of intracellular oxygen, and are nutritionally dependent upon neighbouring cells (see Tabita *et al.*, 1988).

Respiratory protection involves an increase in the cell respiration rate in response to increasing oxygen concentrations (Drozd and Postgate, 1970). The mechanism principally involves the routing of electrons down a branched respiratory chain to one of a number of terminal cytochrome oxidases. At low intracellular oxygen concentrations electrons flow from NADH to oxygen *via* NADH dehydrogenase (site I), the central Q - *b* region (site II) and terminal *c* - *o* (a_1 ?) pathway (site III). However, at high intracellular oxygen concentrations NADH dehydrogenase is no longer energy conserving, and site III is by-passed (electrons flow along the *b* - *d* pathway). The net effect of these changes is to decrease the $\rightarrow H^+/O$ quotient and hence the ATP/O quotient. In order to maintain ATP synthesis the respiration rate increases thus reducing the growth yields ($Y_{substrate}$, Yo_2 and Yco_2) and also decreasing the internal oxygen concentration to a level at which nitrogenase activity is uninhibited (Jones and Redfearn, 1967; Ackrell and Jones, 1971; Jones *et al.*, 1973).

The oxygen problem is the same for bacteroids as free-living cells. However, root nodules contain leghaemoglobin, a role of which is to maintain a low flux of oxygen to respiring bacteroids by reversibly binding oxygen and so reducing the free oxygen concentration (Bergersen and Turner, 1975).

1.6 Carbon nutrition in rhizobia

1.6.1 Free-living rhizobia.

Free-living rhizobia utilize a wide range of carbon substrates for growth, although major differences exist between the genera *Rhizobium* and *Bradyrhizobium*. Members of the genus *Rhizobium* are able to use a broad range of hexoses, pentoses, disaccharides, trisaccharides, sugar alcohols, aromatic and aliphatic organic acids. In contrast, *Bradyrhizobium* utilize hexoses, pentoses, sugar alcohols and a diverse range of aromatic carbon sources, but are unable to use disaccharides, trisaccharides and aliphatic organic acids (see Stowers, 1985). Aliphatic organic acids are transported and oxidised by *Bradyrhizobium japonicum* but do not support growth (Keele *et al.*, 1969; Stowers and Elkan, 1984).

Traditional carbon sources used for culturing rhizobia under laboratory conditions include glucose, mannitol and organic acids, but the survival and growth of rhizobia in the soil may depend on the utilization of aromatic compounds, principally benzoate, catechol, *p*-coumarate, hydroxybenzoate and protocatechuate (Parke and Ornston, 1984; Chen *et al.*, 1984).

Investigations into the mechanisms of carbon transport have only recently been initiated. Virtually no information on the mechanism of sucrose transport exists for rhizobia. Bacteria such as *E. coli* and *Salmonella typhimurium* transport sucrose *via* the phosphotransferase system (PTS) in which the carbohydrate is phosphorylated at the expense of phosphoenolpyruvate (see Postma 1986). However, the existence of PTS-mediated sugar transport in rhizobia is uncertain, but has been discounted for glucose transport on the evidence that no phosphorylated transport products have been detected (Hudman and Glenn, 1980; de Vries *et al.*, 1982; Stowers and Elkan, 1984). Two alternative routes for glucose transport exist, both involving

periplasmic proteins. The first route, already described for *Pseudomonas aeruginosa*, is *via* the oxidation of of glucose to gluconate and then 2-ketogluconate, *via* glucose dehydrogenase and gluconate dehydrogenase, which are then taken up by specific transport systems (Midgely and Dawes, 1973; Roberts *et al.*, 1973; Whiting *et al.*, 1976). This is a low affinity system and repressed under glucose-limited conditions. The second route is *via* a high affinity, periplasmic glucose-binding protein derepressed during glucose-limited growth (Stinson *et al.*, 1977). Two glucose transport systems have been observed in *R. leguminosarum*, differing in their relative affinity for glucose (de Vries *et al.*, 1982).

In the absence of a PTS-mediated glucose transport system the involvement of at least one of the two alternative systems described above in rhizobia is highly likely, since both routes have been identified for *Agrobacterium* (Linton *et al.*, 1986; Cornish *et al.*, 1987; Cornish *et al.*, 1988).

Sucrose uptake is constitutive in *R. leguminosarum* and *R. trifolii* but inducible in *R. meliloti* and cowpea-rhizobia. Glucose uptake is believed to be constitutive in all rhizobia (see Stowers, 1985). Lactose transport almost certainly proceeds *via* a proton symport mechanism which was found to be inducible in all *Rhizobium* sp. studied (Glenn and Dilworth, 1981).

Information on the transport of aromatic carbon sources and sugar alcohols in rhizobia is slight, however, both are active processes, frequently inducible and regulated by catabolite repression (De Vries *et al.*, 1982; Stowers and Elkan, 1984) and/or catabolite inhibition (Ronson and Primrose, 1979).

The presence of an inducible Entner-Doudoroff (ED) pathway in free-living rhizobia is well established (Katznelson, 1955; Ronson and Primrose, 1979; Glenn *et al.*, 1984) and is the major route for hexose metabolism (Keele *et al.*, 1969; Stowers and Elkan, 1984). The absence of phosphofructokinase in numerous strains of *Rhizobium* (Ronson and Primrose, 1979; Glenn *et al.*, 1984; Saroso *et al.*,

1984) indicates that the complete Embden-Meyerhof-Parnas (EMP) pathway is not used by these organisms. Even in *Bradyrhizobium* where the presence of phosphofructokinase and fructose diphosphate aldolase have been reported in glucose grown cells (Stowers and Elkan, 1984) the very low activities, and the absence of phosphofructokinase in cells grown on fructose suggest that the complete EMP pathway is not a significant route of sugar catabolism. The operation of the Embden-Meyerhof-Parnas (EMP) pathway is reported to be strain dependent in both genera (Stowers and Elkan, 1984). EMP activity is also reported to be inducible and present in glucose grown *B. japonicum* cells but not expressed with succinate, gluconate, mannitol, or fructose grown cells (Stowers and Elkan, 1984).

The pentose phosphate pathway (PPP) has only been detected in the genus *Rhizobium* (Keele and Hamilton *et al.*, 1969; Stowers and Elkan, 1984), and its presence or absence has been used as a taxonomic indicator. However, the presence of the PPP has often been assumed following measurements of 6-phosphogluconate dehydrogenase activity. Pentose metabolism in *Bradyrhizobium* proceeds *via* lactone intermediates ultimately yielding pyruvate and acetaldehyde (Pedrosa and Zancan, 1987).

Aromatic carbon metabolism is reported to proceed through a number of routes, but principally is *via* the ß-ketoadipate pathway. Limited studies with *R. trifolii* and *R. leguminosarum* have shown that these pathways are inducible (Chen *et al.*, 1984).

All free-living rhizobia possess a complete TCA cycle (Duncan and Fraenkel, 1979; Ronson and Primrose, 1979; Stowers and Elkan, 1984), the operation of which has been confirmed using radiorespirometry studies with [^{14}C]pyruvate and determining the sequence of [^{14}C]CO_2 release (see Stowers, 1985).

The transport of C_4-dicarboxylic acids has been detected in both *Rhizobium* and *Bradyrhizobium* spp. (Glenn *et al.*, 1980; Finan *et al.*, 1981; McAllister and Lepo, 1983). The C_4-dicarboxylic acid transport (Dct) system appears very similar to that expressed in

bacteroids and capable of transporting succinate, malate fumarate and a range of succinate analogues. The route of C_4-dicarboxylate oxidation in *R. leguminosarum* has recently been shown to operate *via* the TCA cycle and malic enzyme and pyruvate dehydrogenase to generate acetyl-CoA (McKay *et al.*, 1988). A gluconeogenic system for growth on C_4-dicarboxylates by *R. leguminosarum* was proposed by McKay *et al.* (1985) in which oxaloacetate generated from malate was converted to phosphoenolpyruvate (*via* phosphoenolpyruvate carboxykinase), and to fructose 1,6-bisphosphate *via* fructose bisphosphate aldolase activity (through glyceraldehyde 3-phosphate).

1.6.2 Bacteroid carbon metabolism.

Nitrogen fixation by bacteroids requires a considerable input of metabolic energy. Photosynthesis is the primary source of this energy with sucrose as the principal carbon compound transported from photosynthetic leaves and shoots to the roots of leguminous plants.

Although sucrose is the major carbon product imported into root nodules and may also be accumulated by bacteroids (Streeter, 1987), neither disaccharides or monosaccharides are considered primary carbon sources for bacteroid nitrogen fixation, since rhizobia bacteroids do not actively transport sugars (Hudman and Glenn, 1980; Glenn and Dilworth, 1981; DeVries *et al.*, 1982; Reibach and Streeter, 1984), and mutants lacking enzymes of hexose catabolism (glucokinase, fructokinase, phosphoglucoisomerase and pyruvate dehydrogenase; Ronson and Primrose, 1979; Glenn *et al.*, 1984), are still capable of forming effective nitrogen-fixing nodules. However, bacteroids have been shown to accumulate sugars, which enter the cell by passive diffusion (Hooymans and Logman, 1984; Reibach and

Streeter, 1984), but are oxidized at very low rates (Tuzimura and Meguro, 1960). The key enzymes of the Entner-Doudoroff (ED) and some of the Embden-Meyerhof-Parnas (EMP) pathways have been shown to be present in *Rhizobium* and *Bradyrhizobium* bacteroids, but the activities of these enzymes are much lower than in free-living cells (see Elkan and Kuykendall, 1982).

Oxygen uptake in bacteroids is stimulated by a number of carbon substrates, including acetaldehyde, acetate, ethanol, glyoxylate, pyruvate, propionaldehyde, glycerol, benzaldehyde and lactose (Tuzimura and Meguro, 1960; Ratcliffe *et al.*, 1980; Peterson and LaRue, 1981; Hooymans and Logman, 1984). However, these carbon sources have been discounted as major bacteroid substrates, due mainly to their very low rates of oxidation.

The importance of TCA intermediates (principally C_4-dicarboxylates) in the rhizobia-legume symbiosis is well documented (see Stowers, 1985; McDermott *et al.*, 1989). A functional TCA cycle is necessary for nitrogen-fixing activity in bacteroids (Kurz and LaRue, 1975; Stovall and Cole, 1978; Karr *et al.*, 1984), and mutants lacking succinate dehydrogenase or α-ketoglutarate dehydrogenase activity form ineffective nodules (Duncan and Fraenkel, 1979; Gardiol *et al.*, 1982).

C_4-dicarboxylate transport in bacteroids is an active process requiring an energised membrane (Finan *et al.*, 1983; Glenn *et al.*, 1980). The transport of succinate, fumarate and malate is *via* a proton symport mechanism (Glenn *et al.*, 1980; Ronson *et al.*, 1981; Finan *et al.*, 1983). The transport system (Dct) is constitutive and specific for C_4-dicarboxylates (and their associated analogues) (Glenn *et al.*, 1980; Finan *et al.*, 1983). The structural component of the C_4-dicarboxylate transport system is a membrane-bound polypeptide containing approximately 440 amino acid residues (Ronson, 1988).

A functional Dct system is essential for nitrogen fixation activity not only in bacteroids, but also in isolated cells (Finan

et al., 1983) and nodulated plants (Ronson *et al.*, 1981; Glenn and Brewin, 1981; Finan *et al.*, 1983). Dct$^-$ mutants fail to form effective nitrogen-fixing nodules (Ronson *et al.*, 1981), in contrast to mutants with genetic lesions in carbohydrate transport/metabolism (Ronson and Primrose, 1979).

With respect to *Rhizobium* and *Bradyrhizobium* relatively little information has been published on C_4-dicarboxylate metabolism in bacteroids (see McDermott *et al.*, 1989). However, the metabolic routes are believed to be similar to those of free-living cells; *viz* catabolism through the TCA cycle using malic enzyme and pyruvate dehydrogenase activity to generate acetyl-CoA (Mckay *et al.*, 1988).

Few bacteroid TCA cycle enzymes have been characterised. However, Waters *et al.* (1985) characterised *B. japonicum* malate dehydrogenase, which was found to be sensitive to a number of metabolite effectors including NADPH (Emerich *et al.*, 1988), and $NADP^+$-isocitrate dehydrogenase which was sensitive to ATP concentrations (Chandrasekharan and Shethna, 1976). Both enzymes were found to be similar to those of the free-living cells.

In addition to dicarboxylic acids, poly-β-hydroxybutyric acid (PHB) has been reported capable of supporting nitrogen fixation when carbon supply to the bacteroid was reduced (Karr *et al.*, 1984). However, doubts of this ability were expressed by Stam (1986), on the grounds that PHB degradation occurred at oxygen tensions at which nitrogenase activity would be inhibited. PHB is often found in bacteroids where it accounts for up to 50% of the cell dry weight (Wong and Evans, 1971). PHB may either represent a store for excess carbon, or it may serve a regulatory role under oxygen-limited conditions (Senior *et al.*, 1972), where NAD(P)H is channelled into PHB thereby reducing the inhibition of isocitrate dehydrogenase, which would have a direct effect on the flow of carbon through the TCA cycle (Weitzman and Jones, 1968; see McDermott *et al.*, 1989).

1.7 Aims and objectives

The aim of the work presented in this thesis was to investigate the physiology and growth of *Azorhizobium caulinodans* ORS 571 during growth under a variety of nutrient limitations in continuous culture with succinate as the sole carbon source.

The objectives were firstly, to determine the efficiency of growth, measured *via* molar growth yields, cytochrome profiles and $\rightarrow H^+/O$ quotients, under ammonium-assimilating and nitrogen-fixing conditions, and so determine the energy costs of nitrogen fixation in terms of ATP required per mol N_2 fixed. Secondly, to provide information on possible physiological rate-limiting steps associated with growth and nitrogen fixation, through the measurement of enzyme activities, succinate transport and substrate metabolism rates. Thirdly, to investigate the ability of *A. caulinodans* to tolerate oxygen concentrations during growth under nitrogen-fixing conditions far higher than observed for other rhizobia.

CHAPTER 2

Materials and Methods

2.1 Organism

Azorhizobium caulinodans ORS 571 (Dreyfus *et al.*, 1988) was a gift from Dr. J-P. Aubert, Unité de Physiologie Celluaire, Départment de Biochemie et Genetique Moleculaire, Institut Pasteur Paris, France.

A. caulinodans was maintained on Tryptone-Yeast extract agar plates and slopes at 4°C and subcultured monthly.

2.2 Growth Media

2.2.1 Tryptone-Yeast extract agar, nutrient agar and minimal salts agar.

The Tryptone-Yeast extract (TYE) agar used, for the maintenance of *A. caulinodans* contained (gl^{-1}): Difco tryptone, 5.0; Difco yeast extract, 3.0; $CaCl_2.6H_2O$, 1.3; succinic acid, 5.0; Lab M agar, 17.5.

Nutrient agar was used to check for contamination (section 2.3.6) and contained (gl^{-1}): Oxoid nutrient agar, 28.0.

Minimal salts agar was also used as a check for contamination

(section 2.3.6) was of the same composition as the batch medium (2.2.2) and contained (gl^{-1}): Lab M agar, 17.5; elemental carbon (as succinic acid or glucose), 2.4.

The pH of the medium (where appropriate) was adjusted to pH 6.8 using sterile 2M-KOH, and all agar containing media were sterilized at 121°C for 20 min.

2.2.2 Batch culture.

The minimal salts medium used for the growth of *A. caulinodans* was similar to that described by Rye *et al.* (1988), which contained (gl^{-1}): $(NH_4)_2SO_4$, 3.52; KH_2PO_4, 0.68; $MgSO_4.7H_2O$, 0.40; $CaCl_2.2H_2O$, 0.0012; Iron citrate, 0.0268; Nicotinic acid, 0.02; Biotin, 0.001; HEPES buffer, 4.77, and 2.5 ml trace element solution which contained (mgl^{-1}): $MnSO_4.7H_2O$, 0.56; $ZnSO_4.7H_2O$, 0.72; $CuSO_4.5H_2O$, 0.625; $CoCl_2.6H_2O$, 0.297; $Na_2MoO_4.2H_2O$, 0.312; KI, 0.207; H_3BO_3, 0.078. The medium was supplemented with succinic acid (6 gl^{-1}) or other carbon sources (2.4 g carbon l^{-1}) depending on experimental conditions.

The pH of the medium was adjusted to pH 6.8 using 2M-KOH, and sterilized at 121°C for 25 minutes.

2.2.3 Continuous culture.

The medium used for carbon limitation was of the same mineral salts composition as the batch culture medium except that HEPES buffer was omitted and the succinic acid was increased to 7.2 gl^{-1} (equivalent to 2.92 g carbon l^{-1}). The DOT was maintained at 30% air saturation.The media used for phosphate, sulphate and oxygen limitation were of the same mineral salts composition as for carbon limitation except for the following changes:

(a) Phosphate limitation (gl^{-1}) : succinic acid, 10.0; KH_2PO_4, 0.109; K_2SO_4, 3.65. Total P = 0.025 gl^{-1}. The DOT was maintained at 30% air saturation.

(b) Oxygen limitation (gl^{-1}) : succinic acid, 4.0-25.0.

(c) Sulphate limitation (ammonium assimilating) (gl^{-1}) : succinic acid, 10.0; NH_4Cl, 2.85; $MgSO_4.7H_2O$, 0.139; $MgCL_2.6H_2O$, 0.162. Total S = 0.018 gl^{-1}. The DOT was maintained at 30% air saturation.

(d) Sulphate limitation (ammonium free) (gl^{-1}) : succinic acid, 10.0; Iron citrate, 0.05; $Na_2MoO_4.2H_2O$, 0.08.

The 40L bulk medium was sterilized at 121°C for 35 min. All continuous culture media contained polypropylene glycol 2025 (BDH Chemicals Ltd, Poole, Dorset) as an antifoam at a concentration of 0.25 ml l^{-1}. The antifoam was autoclaved separately prior to mixing with the bulk medium.

2.3 Growth conditions

2.3.1 Batch culture.

Cells were grown in baffled shake flasks of 250 or 500 ml volume containing 50 or 150 ml medium respectively, inoculated from TYE agar slopes. Flasks were incubated at 30°C and agitated continuously on an orbital shaker at 120 rev. min^{-1}.

2.3.2 Continuous culture.

For preparative work carried out at Leicester University, *A. caulinodans* was grown in a bottom-driven LH 500 series fermenter (LH Engineering Ltd, Stokes Poges, U.K.), with a working volume of 900 ml. Medium was pumped into the the fermenter from a 20l reservoir using a 1200 series Varioperpex peristaltic pump (Pharmacia LKB Instruments Ltd, Milton Keynes, U.K.). Aeration was 600 ml air min^{-1}, sparged at the base of the fermenter directly beneath the impeller drive shaft. The culture was agitated at 600 rev. min^{-1} with two three-blade impellers. These conditions were sufficient to maintain the dissolved oxygen tension (DOT) at $\geq$30% air saturation as measured using a galvanic oxygen electrode (LH Engineering).

During oxygen-limited growth the culture density was maintained between 0.5-0.8 g cell dry weight l^{-1} by reducing the air flow to 75 ml min^{-1} and decreasing the agitation to 250 rev. min^{-1}. These conditions maintained the dissolved oxygen tension at <1% of air saturation.

The culture pH was maintained at the desired value (normally 6.8 ±0.02) by the addition of 2M-KOH to the fermenter. Alkali addition was controlled *via* an autoclavable pH reference electrode (Russell Instruments Ltd, Auchtermuchty, Scotland), linked to an LH 500 series pH control module.

The culture temperature was maintained at 30 ±0.05°C using a potentiometric controller in conjunction with a resistance thermometer coupled to an electric heating finger immersed in the culture.

For growth yield studies carried out at Shell Research Ltd, *A. caulinodans* was grown in a 3 litre Biotech fermenter, the head plate of which was modified to take a direct drive assembly. The medium was pumped into the fermenter from a 40 litre reservoir using an LKB 1200 series Varioperpex peristaltic pump. The culture was stirred

continuously at 600 rev. min^{-1} using two six-blade Rushton type impellers, positioned close to the base and three-quarters up the drive shaft respectively. Culture pH was maintained at the desired value (normally 6.8 ±0.02) using an autoclavable Russell pH electrode coupled to a Turnbull pH control system (Turnbull Control Systems Ltd, Worthing, U.K.). The temperature of the culture was maintained at 30.0 ±0.5°C using a potentiometric controller connected to a resistance thermometer, and coupled to a 120W electric heating finger located on the base plate of the fermenter. A chilled water cooling finger was always present.

Aeration of the culture was achieved by sparging 600 ml min^{-1} air-nitrogen mix into the fermenter through an inlet port positioned directly beneath the drive shaft. The composition of the air-nitrogen mix was dependent upon the required dissolved oxygen tension in the fermenter.

2.3.3 Dissolved oxygen control.

The dissolved oxygen tension (DOT), in the Biotech fermenter was maintained and controlled using an Ingold polarographic autoclavable oxygen probe (Ingold A.G, Urdorf, Switzerland), connected in series to a Chemap conditioning-amplifier (Alfa Laval A.G, Mannendorf, Switzerland). A complete description of this system is given in Chapter 5 section 5.2.1.

2.3.4 Fermenter inoculation.

Inoculation of both the LH and Biotech fermenters was achieved by the sterile transfer of exponentially-growing cells from shake flasks cultured on the same medium as that to be used in the fermenter. The volume transferred was 20% of the final fermenter vol-

ume, and was transferred *via* a sterile connector to a port located on the head-plate of the fermenter.

2.3.5 Sampling methods.

Samples not exceeding 25 ml volume for routine analysis were taken through a spring-loaded port located on the base-plate of the Biotech fermenter. Samples for steady-state analysis were collected in a sterile, ice-cooled graduated measuring cylinder (500 ml volume) connected to the fermenter effluent line.

2.3.6 Fermenter contamination check.

Samples of culture ($\simeq$ 5 ml) were taken from the fermenter at regular intervals and assessed for contamination. Samples were incubated on Tryptone-Yeast extract (TYE) agar plates (pH 6.8), and nutrient agar plates. Good growth and the presence of a single colony morphology on the TYE agar plates, and no growth on the nutrient agar was indicative of *A. caulinodans*. The samples were also incubated on minimal salts media agar plates (section 2.2.1). Good growth and a single colony morphology on the succinate containing agar plates, in conjunction with no growth on the glucose containing plates was indicative of *A. caulinodans*.

All agar plates were incubated at 30°C for up to five days. The culture was also examined daily using a Wild M20 light microscope (Wild Heerburg AG., Switzerland), under oil immersion at a magnification of 1000 fold.

2.4 Measurements of fermentation parameters

2.4.1 Measurement of gaseous oxygen, carbon dioxide and nitrogen in the fermenter gas streams.

The inlet and outlet gas compositions of steady-state cultures were measured at seven-minute intervals over a ninety six hour time period using an MM80 VG Gas Analysing Mass Spectrometer (VG Gas Analysis Ltd, Middlewich, U.K.). The mass spectrometer was automatically calibrated every six hours using a standardized gas mix of carbon dioxide, oxygen, nitrogen and argon (Cryoservice Ltd, Blackpole Rd., Worcester, U.K.).

2.4.2 Gas flow rate.

The inlet gas flow rate to the fermenter was calibrated and measured using a Hi-tec Mass Flow Controller (this is comprehensively described in chapter 5.2.1).

2.4.3 Inlet gas temperature.

The inlet gas temperature of the fermenter was measured using a standard mercury thermometer plumbed into the inflowing gas line.

2.4.4 Inlet and outlet gas pressure.

The inlet gas to the fermenter was at atmospheric pressure and

was measured using a Fortin-type barometer. The pressure of the outlet gas was measured using a mercury manometer plumbed into the outlet gas line.

2.4.5 Liquid flow rates.

The flow rate of medium into the fermenter was measured using a calibrated arm on the line from the medium reservoir. The time taken to pump 20 ml of medium from the side arm into the fermenter was measured three times and a mean value was calculated. Culture outflow from the fermenter was measured by collecting effluent in a graduated measuring cylinder for 60 min.

2.4.6 Culture volume.

The culture volume was measured using a calibrated volume scale on the sight glass of the fermenter, following completion of all steady-state measurements and with the drive motor disengaged.

2.4.7 Culture density.

Culture samples (30 ml) were taken from the fermenter and centrifuged at 40,000g for 15 min at 5°C. The cell pellet was washed three times with distilled water, resuspended in the same and dried to a constant weight at 98°C.

No appreciable cell lysis occurred during this procedure, as measured by the release of protein into the washings using the modified Lowry assay of Peterson (1977), with bovine serum albumin as the standard.

2.4.8 Culture absorbance.

The culture absorbance (OD_{600}) was measured at 600nm using a Pye Unicam SP6-500 U.V spectrophotometer (Pye Unicam Ltd, Cambridge, U.K). For *in vitro* experiments a cell dry weight /optical density calibration curve for cells from each different nutrient limitation studied was constructed, and used to convert cell optical density (OD_{600}) readings to cell density (gl^{-1}).

2.4.9 Total organic carbon analysis.

The total organic carbon contents of growth media, cell cultures and culture supernatants were measured using a Shimadzu Total Organic Carbon Analyser TOC 500 (Shimadzu Corp., Kyoto, Japan), as described by Linton *et al.* (1977).

2.4.10 Elemental carbon, hydrogen, phosphorous, nitrogen and oxygen analysis.

The percentage of elemental carbon, hydrogen, phosphorous, oxygen and nitrogen present in freeze-dried samples of bacterial cells was determined using a Hewlett-Packard 185 Elemental Analyser (Hewlett-Packard Co., Sunnyvale, CA 94806, U.S.A.) according to the manufacturer's instructions. These analyses were performed by the Analytical Chemistry Division, Shell Research Ltd, Sittingbourne, Kent, U.K.

2.4.11 Determination of molar growth yields.

Bacterial molar growth yields from succinate and oxygen consumption and carbon dioxide production (Ysuccinate, Yo_2 and Yco_2; g dry weight mol^{-1}) were calculated from the *in situ* rates of succinate and oxygen consumption (qSuccinate and qO_2), and carbon dioxide evolution (qCO_2; mmol h^{-1} [g cell dry weight]$^{-1}$) from steady-state cultures. Culture steady-state was attained only after at least five complete volume changes in the fermenter had taken place during which the culture density and all measurable parameters remained constant. A computer programme developed in the Fermentation and Microbiology Division, Shell Research Ltd, Sittingbourne, Kent, was used for the calculation of molar growth yields from the measured parameters (see Appendix 1).

Maximum molar growth yields (Y^{max}succinate, $Y^{max}O_2$ and $Y^{max}CO_2$), and maintenance coefficients (*M*succinate, Mo_2 and Mco_2), were calculated from plots of qSuccinate and qO_2 against the dilution rate (D,h^{-1}), where the Y^{max} equalled the reciprocal of the slope, and *M* was the q value at zero growth rate (Pirt, 1975; Fieschko and Humphrey, 1984).

The dilution rate was calculated from the rate of inflowing media (ml h^{-1}) divided by the fermenter working volume (l).

2.4.12 Analysis of culture supernatants.

Culture supernatants were prepared from fermenter samples (30 ml) by centrifugation at 40,000g for 15 min. Samples were assayed for succinic acid and other organic acids using High Performance Liquid Chromatography (HPLC) and elemental analysis using Plasma Emission Spectroscopy. These analyses were kindly performed by Mr. M. Platten and Mr. L.J. Barnes respectively, in the Fermentation and

Microbiology Division, Shell Research Ltd, Sittingbourne, Kent. U.K.

2.5 *In vitro* analysis

2.5.1 Preparation of washed cell and broken cell suspensions.

Cultures were harvested by centrifugation at 30,000g for 15 min at 5°C. The cell pellet was washed twice with 20-mM HEPES/KOH buffer pH 7.0 at 5°C, and finally resuspended in the same buffer to a cell density of approximately 6 g cell dry weight l^{-1}.

Broken cell suspensions were prepared by sonicating washed cell suspensions (cooled on ice), at 10μ amplitude for four 30s periods with 1.5 min intervals between each period.

2.5.2 Polarographic measurement of bacterial respiration rates.

For the measurement of the potential respiration rate (potential qO_2), a 10 ml sample of culture was rapidly removed from the fermenter. A 500 μl aliquot was immediately injected into the reaction chamber of a Clark-type oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K). The reaction chamber contained 3.5 ml succinate-minimal salts medium minus the nitrogen source (see 2.2.2) at pH 7.0 and 30°C.

Respiration rates of washed cells suspensions oxidising various substrates were also measured in a Rank oxygen electrode at 30°C. Washed cell suspensions were prepared from cultures grown under different nutrient-limitations as described above. The reaction mix

(final volume 4.0 ml) contained 3.8 ml HEPES/KOH buffer pH 7.0, and 0.1 ml substrate (10-mM oxidizable carbon source or 5 mM-ascorbate/1 mM-TMPD). A 0.1 ml aliquot of cell suspension (0.5-3.0 mg cell dry weight), was injected into the reaction chamber 1 min prior to the addition of the substrate.

For experiments that required the use of metabolic inhibitors (e.g. FCCP), cell suspensions were equilibrated for 1 min in the presence of the inhibitor prior to the addition of substrate. All measured rates of oxygen consumption were corrected for endogenous respiration and/or substrate autoxidation. The concentration of oxygen in air saturated HEPES/KOH buffer, pH 7.0 at 30°C, was taken as 230 μM.

2.5.3 Measurement of bacterial cytochrome content.

Cytochrome difference spectra were recorded at room temperature using a Perkin Elmer Lambda-5 split beam spectrophotometer (Coleman Instruments Division, Illiois 60153, U.S.A), with a volume of 1 ml, a 1 cm path length and a slit width of 2 nm.

Washed cell suspensions (6 g cell dry weight l^{-1}) were prepared as previously described. Reduced-*minus*-oxidized spectra were recorded following oxidation of the contents of the reference cuvette, using 5 μl of a saturated ferricyanide solution, and reduction of the contents of a sample cuvette using a few grains of sodium dithionite. All spectra were referenced to a oxidised-*minus*-oxidised baseline.

Concentrations of cytochromes were estimated from the peak to trough absorbence differences using the appropriate molar absorption coefficients (Carver and Jones, 1973; Jones and Poole, 1985; Cornish *et al.*, 1987). Cytochrome aa_3 was measured from the alpha-peak (600 nm) to a trough on the red side ($\sim$620nm) using a millimolar extinction coefficient of 11.7 mM^{-1} cm^{-1}. Cytochrome *b* was measured from

the alpha-peak (555 nm) to a trough on the red side (~572nm) using a millimolar absorption coefficient of 17.5 mM^{-1} cm^{-1}. Cytochrome *c* was measured from the alpha-peak (550 nm) to a trough on the blue side (~538nm), using a millimolar absorption coefficient of 17.3 mM^{-1} cm^{-1}. Cytochrome *d* was measured from the peak at 630nm to a position 15nm towards the blue, using a millimolar absorption coefficient of 19.0 mM^{-1} cm^{-1}.

Second order derivatives of these spectra were measured using a $\Delta\lambda$ of 3nm.

2.5.4 Measurement of dicarboxylate uptake and metabolism.

Washed cell suspensions, prepared as previously described, were incubated in the reaction chamber of a Rank oxygen electrode. The reaction mix (final volume 1 ml; 30°C) contained 0.895 ml 20-mM HEPES/KOH buffer pH 6.8, to which was added 0.1 ml cell suspension (10 g cell dry weight l^{-1}). The reaction mix was equilibrated for 1 min prior to the addition of 5 μl (250 nmol) [2,3-^{14}C]succinic acid at a specific activity of 1.35 mCi $mmol^{-1}$. The radio-labelled succinate was obtained by diluting stock [2,3-^{14}C]succinic acid at a specific activity of 40-42 mCi $mmol^{-1}$, with the appropriate amount of unlabelled potassium succinate.

Samples (100 μl) were withdrawn at 15s intervals and were rapidly filtered under vacuum through 0.45 μm pore nitrocellulose filters (Sartorius Gmbh, 4000 Göttingen, West Germany). The filter was immediately washed twice with 1.5 ml cold 20-mM HEPES/KOH buffer, pH 7.0, and plunged directly into 3 ml Fisofluor 3 scintillation fluid (Fisons Scientific Apparatus Ltd, Loughbourgh, U.K), prior to counting in a Hewlett Packard Tri-carb scintillation counter (Hewlett-Packard Co., Sunnyvale, CA 94806, U.S.A). This technique is referred to as the "Wet Method". In some experiments the

filters were placed under a heating lamp and dried for 20 min, this technique is referred to as the "Dry Method".

[2,3-^{14}C]maleate (cis-fumarate) was used as a non-metabolizable analogue of succinic acid in a number of experiments. As [2,3-^{14}C]maleate was not available commercially it was prepared by allowing [2,3-^{14}C]maleic anhydride to undergo spontaneous hydrolysis. A solution of [2,3-^{14}C]maleic anhydride was allowed to stand at room temperature for 6h in order to allow complete conversion. The resultant [2,3-^{14}C]maleic acid was then diluted with the appropriate amount of unlabelled potassium maleate to the same specific activity as the [^{14}C]succinate described above.

Rates of [2,3-^{14}C] dicarboxylic acid uptake were measured using duplicate samples and quantified using an appropriate quench correction factor.

The disappearance of [2,3-^{14}C]succinate or maleate from the reaction mix was measured by withdrawing 100 μl samples at various time intervals, centrifuging them at 10,000g for 1 min in a microcentrifuge, then transferring 20 μl of the supernatant to a nitrocellulose filter which was dried under a heating lamp and quantified by scintillation counting.

2.5.5 Measurement of dicarboxylate accumulation.

Washed cell suspensions, prepared as previously described, were incubated in the reaction chamber of a Rank oxygen electrode in the same manner as described in section 2.5.4. Samples (100 μl) were withdrawn after 10s (following the addition of the radio-labelled substrate), rapidly filtered under vacuum through 0.45 μm pore nitrocellulose filters, washed twice with 1.5 ml cold 20-mM HEPES/KOH buffer and plunged directly into 2.5 ml (approx.) boiling distilled water. The whole procedure was replicated 4 times and the pooled aqueous extracts were freeze-dried and finally re-dissolved in

2 ml distilled water.

Dicarboxylates and other intermediary metabolites in the extracts were separated by paper chromatography using Whatman No.1 paper. The equivalent of 0.2 mg cell dry weight extract (approx. 50 μl) was loaded on to the paper. 5 μl (250 nmol) [2,3-^{14}C]succinate and [2,3-^{14}C]maleate (specific activity 40-42 mCi mmol^{-1}) were used as standards. Unlabelled standards were also run as appropriate. The mobile phase solvent system contained n-propanol/aqueous-ammonia/distilled water (6:3:1, by vol). Autoradiographic images were recorded from the dried chromatograms using Kodak X-OMAT AR diagnostic film (Eastman Kodak Co., Rochester, New York, U.S.A.).

The [^{14}C]-containing spots present on the chromatogram were quantified by cutting the chromatogram into sequential strips (2.5 cm wide and 1 cm long), which were placed in Fisofluor 2 and quantified by scintillation counting.

2.5.6 Polyacrylamide gel electrophoresis of bacterial proteins.

Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), of cell proteins was carried out using 7-20% (w/v) gradient polyacrylamide gels according to the method of Laemmli (1970).

Samples of cells (approx. 25 μg dry weight per track), were boiled for 15s in dissolving buffer (1 ml) that contained 0.05 ml ß-mercaptoethanol, 0.6 ml cell sample and 0.35 ml sample buffer pH 6.8 (which contained gl^{-1}: glycerol, 250.0; Tris/HCl, 21.8; EDTA, 0.18; sodium dodecyl sulphate, 57.0), prior to loading on the gel. The stacking gel contained (ml,l^{-1}): Tris (0.5M) + sodium dodecyl

sulphate (0.4% v/v), 2.5; acrylamide soln. (300.0 gl^{-1} acrylamide, 8.0 gl^{-1} bis-acrylamide), 1.5; distilled H_2O, 6.0; ammonium per sulphate (33.0 gl^{-1}), 0.09; TEMED, 0.01. The separating gel contained (ml,l^{-1}): Tris/HCl (1.5M) + sodium dodecyl sulphate (0.4% v/v), 7.0; acrylamide soln. (300.0 gl^{-1} acrylamide, 8.0 gl^{-1} bis-acrylamide), 13.0; super *q* H_2O, 7.75; ammonium persulphate (33.0 gl^{-1}), 0.17; TEMED, 0.014; glycerol, 0.35. The running buffer contained (gl^{-1}): Tris/HCl, 6.0; glycine, 28.8; sodium dodecyl sulphate, 1.5. The gel was run with a voltage of 500v and a current of 40 mAmps.

A molecular weight marker kit, MW-SDS-70L (Sigma Chemical Co. Ltd., Poole, U.K.), was used to determine protein molecular weights between 14,000 and 70,000 Daltons. The gels were stained for protein with Kenacid-R (Weber and Osbourne, 1975).

Haem-associated proteins were characterised using the same method as described above, but with approximately 250 μg dry weight per track and in the absence of ß-mercaptoethanol from the dissolving buffer. Gels were stained for haem-associated peroxidase activity with TMBZ according to the method of Thomas *et al.* (1976).

2.5.7 Preparation of periplasmic shock fluid.

Periplasmic extracts were prepared by osmotically shocking cell suspensions according to the method of Cornish *et al.* (1988). Cells were washed three times using 30mM Tris/HCl (pH 8.0) and resuspended in 30 mM Tris/HCl (pH 8.0) containing 20% (w/v) sucrose to give a cell density of 10g dry weight l^{-1}. The cell suspension was then shaken gently at 30°C for 10 min during which time EDTA was added (as 50 μl aliquots of a 100 mM stock solution) to give a final concentration of 5 mM. The cells were centrifuged (15,000g for 15 min) resuspended to the initial volume in cold distilled water (i.e. subjected to osmotic shock) and stirred on ice for 10 min. After this

time the cells were centrifuged (15,000g for 15 min) and the supernatant (shock fluid) removed. The protein content of the shock fluid, cells and shocked cells were analysed using SDS-PAGE as described above.

2.5.8 Enzyme activities in broken cell preparations.

2.5.8.1 Succinate dehydrogenase activity.

Succinate dehydrogenase activity in broken cells was measured spectrophotometrically at 30°C by following the change in absorbence at 600nm due to the reduction of 2,6-DCPIP in the presence of reduced PMS. The enzyme activity was quantified using a 2,6-DCPIP millimolar absorption coefficient of 19.1 mM^{-1} cm^{-1}, and the results expressed as: nmol succinate min^{-1} (mg cell dry weight)$^{-1}$.

The reaction mix (final volume 1 ml) contained: 2,6-DCPIP, 25 μl (2mM stock soln); PMS, 100 μl (4.3mM stock soln); KCN, 20 μl (40mM stock soln); HEPES/KOH buffer pH 7.0, 100 μl (1M stock soln.); cell preparation, 5 μl (0.075 gl^{-1} dry weight); distilled water, 650 μl; potassium succinate, pH 7.0, 100 μl (0.1M stock soln).

The reaction was initiated by the addition of potassium succinate to the test cuvette, and the progressive reduction of 2,6-DCPIP followed for at least 4 min. The reference cuvette contained the reaction mix with the exception of 2,6-DCPIP and potassium succinate. All rates were corrected for auto-oxidation and the procedure repeated at least 3 times per individual determination.

2.5.8.2 Malate dehydrogenase activity.

Malate dehydrogenase activity in broken cells was measured spectrophotometrically at 30°C in the reverse direction by following the change in absorbence at 340nm due to the oxidation of NADH following the conversion of oxaloacetate to malate. The activity was quantified using an NADH millimolar extinction coefficient of 6.22 mM^{-1} cm^{-1} (Bergmeyer, 1974).

The reaction mix (final volume 1 ml) contained: Tris/HCl buffer, pH 8.0, 820 μl (0.1M stock soln); $MgCl_2$, 50 μl (0.1M stock soln); cell preparation (0.1 gl^{-1} dry weight), 20 μl; NADH, 20 μl (5.5mM stock in Tris/HCl buffer pH 8.0); oxaloacetate, 10 μl (0.1M stock in Tris/HCl buffer pH 8.0).

The reaction was initiated following the addition of OAA to the test cuvette 2 min after the addition of NADH. The reference cuvette contained the reaction mix with the exception of NADH and oxaloacetate. Malate dehydrogenase rates were corrected for NADH oxidation prior to the addition of OAA.

2.5.8.3 NADH oxidase activity.

NADH oxidase activity in broken cells was measured spectrophotometrically at 30°C by following the change in absorbence at 340nm due to the oxidation of NADH.

The reaction mix (final volume 0.99 ml) contained: Tris/HCl buffer, pH 8.0, 820 μl (0.1M stock soln.); $MgCl_2$, 50 μl (0.1M stock soln); cell preparation (0.1 gl^{-1} dry weight), 20 μl; NADH, 20 μl (5.5 mM stock in Tris/HCl buffer pH 8.0).

The reaction was initiated following the addition of NADH to the test cuvette. The reference cuvette contained the reaction mix with the exception of NADH.

2.5.9 Measurement of poly-ß-hydroxybutyrate content of cells.

The poly-ß-hydroxybutyrate (PHB), content of bacterial cells was measured in freeze-dried bacterial preparations according to the method of Law and Slepecky (1961).

Purified bacterial PHB (0.5g) (Sigma Chemical Co. Ltd), was hydrolysed to crotonic acid in 20 ml concentrated sulphuric acid for 10 min at 60°C, and measured for crotonic acid acid content spectrophotometrically at 235 nm, against a crotonic acid standard solution, and a linear calibration graph constructed. Freeze-dried bacterial cells (0.25g) were similarily hydrolysed in 20 ml concentrated sulphuric acid, centrifuged at 40,000g for 15 min and the supernatant assayed for PHB referenced to the calibration graph.

2.5.10 Presentation of results.

Where appropriate, results are presented as the mean ±SEM with the number of determinations in parentheses. Data lines were fitted to data points by linear regression analysis using Sigmaplot version 3.1 software (Jandel Scientific Company, Corte Madera, California, U.S.A.), and an Amstrad PC1640 personal computer (Amstrad Plc., Brentwood, Essex, U.K.).

2.6 Chemicals

All chemicals unless otherwise stated were obtained from BDH Ltd., Dagenham, U.K. Succinic acid was obtained from Fluka Chemicals Ltd., Glossop, U.K. All radio-labelled chemicals were obtained from Amersham International plc, Amersham, U.K. Chemicals used for inhibitor and enzymic assay were obtained from Sigma Chemical Company, Poole U.K.

2.6.1 Chemical abbreviations

ATP	:	Adenosine 5′-triphosphate.
pCMB	:	p-Chloromercuribenzoate.
pCMBS	:	p-Chloromercuribenzene sulphonate.
DCPIP	:	2,6-Dichlorophenol indophenol.
DMF	:	N,N-Dimethylformamide.
FCCP	:	Fluorocarbonylcyanide 3-chlorophenylhydrazone
HEPES	:	N-2-Hydroxyethylpiperazine-N-2-ethane sulphonate.
NAD^+	:	Nicotinamide-adenine dinucleotide (oxidized).
NADH	:	Nicotinamide-adenine dinucleotide (reduced).
NEM	:	N-ethylmaleimide.
PMS	:	Phenazine methosulphate.
TMBZ	:	3,3′,5,5′-Tetramethylbenzidine.
TMPD	:	N,N,N′N′-Tetramethyl-p-phenylenediamine.
TTFA	:	2-Thenoyltrifluroacetone.

CHAPTER 3

Growth energetics of *A. caulinodans*

3.1 Introduction

It is well established that carbohydrate supply from photosynthate is of major importance for symbiotic activity (Hardy and Havelka, 1975; Bergersen and Turner, 1980). Studies have shown that sucrose is actively transported in root nodules, and relatively high levels of sucrose and glucose exist in the cytosol of nodule cells (Davis and Nordin, 1983). However, despite the wide array of potential carbon sources available to and oxidised by bacteroids (see Stovall and Cole, 1978; Ratcliffe *et al.*, 1980), organic acids, principally TCA intermediates (succinate, malate and fumarate), are probably the major source of energy for the bacteroid (Ronson and Primrose, 1979; Ronson *et al.*, 1981; Glenn and Brewin, 1981; Gardiol *et al.*, 1982; Finan *et al.*, 1983). In addition to providing the bacteroid with a source of energy, the metabolism of organic acids (principally succinate) has also been suggested as a controlling/initiating factor involved in nodule development and bacteroid differentiation (Duncan and Fraenkel, 1979, Gardiol *et al.*, 1982; Urban and Bechtel, 1984).

The supply of photosynthate to root nodules by the plant host has been suggested as a potential control point for regulating nitrogen fixation (Silsbury, 1977; Stouthamer, 1977). Carbon flux to root tissue is not constant and it is possible that bacteroids may encounter periods of carbon-excess and carbon-limitation. Carbon-

excess (energy-excess) conditions may result in regulation of the substrate uptake system, the induction of so called "energy spilling" reactions (Neijssel and Tempest, 1976) and may lead to formation of PHB, polysaccharide *etc;* in order to regulate internal substrate and energy levels, and alleviate the possible inhibitory effects of excess ATP production. The synthesis of energy- and carbon-rich products, especially extracellular polysaccharides are often observed in free-living rhizobia (Zvenhuizen, 1971,1984; Hollingsworth *et al.*, 1985). Polysaccharide production is also important during the nodulation process in that the major surface polysaccharides of *Rhizobia* are thought to be involved in host/symbiont recognition processes and in the binding of the *Rhizobia* to the host root hair, and so may serve a dual function (Bhagwat and Thomas, 1984; Olivares *et al.*, 1984).

The rate of oxygen diffusion through nodule tissue and its availability to bacteroids may influence the composition of the respiratory chain (as in many free-living rhizobia), and so affect respiratory efficiency and energy conservation. Under oxygen-limited (energy-limited) conditions free-living rhizobia often synthesize large amounts of poly-β-hydroxybutyrate (accounting for up to 50% of the cell dry weight) (Wong and Evans, 1971), which may act as a sink for excess NADH. Accummulation of large amounts of PHB has also been reported to occur in bacteroids under certain conditions (Forsyth *et al.*, 1958), and it has been suggested that it can serve as a substrate to support nitrogen fixation when photosynthate supply becomes limiting (Bergersen, 1984).

The values of $Y^{max}_{substrate}$ and $Y^{max}_{O_2}$ for rhizobia are often lower than values reported for other similar heterotrophic bacteria having two or three sites of proton translocation with oxygen as the terminal electron acceptor. The nature of the terminal oxidase present can strongly influence energy conservation; the replacement of either cytochrome oxidase *o* or aa_3 by cytochrome oxidase *d* in cells of *R. trifolii* under oxygen-limited conditions in the presence

of cytochrome *c* resulted in a decrease in the observed Yo_2 from 31.7 to 22.8 g cell dry wt mol^{-1} (De Hollander, 1981). Ratcliffe *et al.* (1983) reported $Y^{max}glucose$ and $Y^{max}o_2$ values of 80.3 and 25.6 g dry wt mol^{-1} respectively for *R. leguminosarum* grown under glucose limitation, a low calculated $Y^{max}o_2$ value of 33.0 g dry wt mol^{-1} can be calculated from the data of De Hollander and Stouthamer (1979) when grown carbon-limited on mannitol, and Stam *et al.* (1984) reported a Yo_2 of 27.0 g dry wt mol^{-1} for *A. caulinodans* grown under succinate-limited conditions ($D=0.10h^{-1}$).

The reasons for the low observed growth yields could be a result of high rates of carbon dioxide fixation, the production of "overflow" metabolites, low P/O quotients, or a high $\rightarrow H^+/ATP$ quotient, nitrogen fixation and a high permeability of the coupling membrane to protons have all been suggested, the latter almost certainly being so for *R. leguminosarum* (Ratcliffe *et al.*, 1983).

In this chapter the efficiency of energy conservation for *A. caulinodans* was measured when grown in minimal salts medium in continuous culture under succinate, sulphate and oxygen limitation. Molar growth yields on succinate, oxygen and carbon dioxide were correlated with the cytochrome content of the bacteria, characterised by spectral analysis and inhibitor studies. The effects of nutrient limitation on succinate dehydrogenase, malate dehydrogenase and NADH oxidase activities and the synthesis of PHB were also measured.

3.2 Results

3.2.1 Growth substrates.

The ability of *A. caulinodans* to utilize a possible 26 carbon substrates for growth was tested using defined medium in batch culture (as described in Materials and Methods). It was found that 8 compounds (acetate, fumarate, gluconate, 2-ketogluconate, lactate, malate, pyruvate and succinate), were used as carbon and energy sources for growth, whereas, the remaining 18 compounds (arabinose, citrate, fructose, galactose, glucose, glycerol, isocitrate, maleate, maltose, mannitol, propionate, rhamnose, sorbitol, sucrose, trehalose, xylitol and xylose) did not support growth (Table 3.1).

The cells appeared to assimilate only a limited range of organic acids, and not to assimilate sugars or alcohols. The results of a more recent exhaustive study by Dreyfus *et al.* (1988), detailing the utilization of 151 carbon sources by 20 isolates of *A. caulinodans* ORS spp. confirmed the results described above except that approximately 90% of the tested strains were able to grow on glucose, citrate, propionate and maleate.

The inability of *A. caulinodans* to use glucose (or other sugars), in this study separated it from all other *Rhizobium* and *Bradyrhizobium* spp., and presumably reflects an inability to transport and/or metabolize the sugar.

To investigate whether this was due to the presence of an inactive, apoenzyme form of the quinoprotein glucose dehydrogenase (GDH), 20 μM pyrroloquinoline quinone (PQQ), was added to cell cultures with glucose as the sole carbon source. The presence of PQQ did not stimulate growth on glucose, suggesting either that *A. caulinodans* did not possess GDH (in contrast to several closely

Table 3.1. The growth of *A. caulinodans* in batch culture at 30°C, pH 6.8 on various carbon substrates. The growth medium was as described in Materials and Methods and contained 2.41 g carbon l^{-1}.

Growth substrate			
Acetate	+	Malate	+
Arabinose	-	Maleate	-
Citrate	-	Maltose	-
Fructose	-	Mannitol	-
Fumarate	+	Propionate	-
Galactose	-	Pyruvate	+
Gluconate	+	Rhamnose	-
Glucose	-	Sorbitol	-
Glucose-6-P	-	Succinate	+
Glycerol	-	Sucrose	-
Isocitrate	-	Trehalose	-
2-Ketogluconate	+	Xylitol	-
Lactate	+	Xylose	-

+ ; growth.

- ; growth not detected.

related Gram-negative bacteria), or perhaps that maintenance and subcultivation of the stock culture on tryptone-yeast extract medium inadvertently selected for a strain that had lost the ability to grow on glucose.

The preference for organic acids as carbon substrates, together with the lack of sugar utilization (this study; also Dreyfus *et al.*, 1988), suggested that free-living *A. caulinodans* may represent an intermediary state in bacteroid development, in terms of carbon transport and metabolism (Rawsthorne *et al.*, 1980; Saroso *et al.*, 1984; Stowers, 1985).

3.2.2 Cellular pleomorphy.

Following growth in batch cultures on succinate-containing defined media *A. caulinodans* occasionally underwent morphological changes, which may have resulted from a failure of the rod-shaped cells to divide normally, leading to V, T, Y and other irregular shapes (Fig 3.1). The cells also appeared to accumulate an intracellular storage product, probably poly-ß-hydroxybutyrate (PHB).

These changes did not occur during the exponential growth phase, but more commonly appeared approximately ten hours post inoculation, and were always associated with cessation of growth. The changes were not always uniform throughout the culture suggesting that the cells were not adversely influenced by pH or osmotic effects.

Cellular extracts of *A. caulinodans* (prepared by sonication and filtration through 2 μm pore sterile filters, of the pleomorphic cells), did not induce pleomorphic changes in non-differentiated cells *per se* when added to liquid or solid media. This result suggested that the changes were probably not caused by a transferable factor, for example a bacteriophage, as reported for Chickpea *Rhizo-*

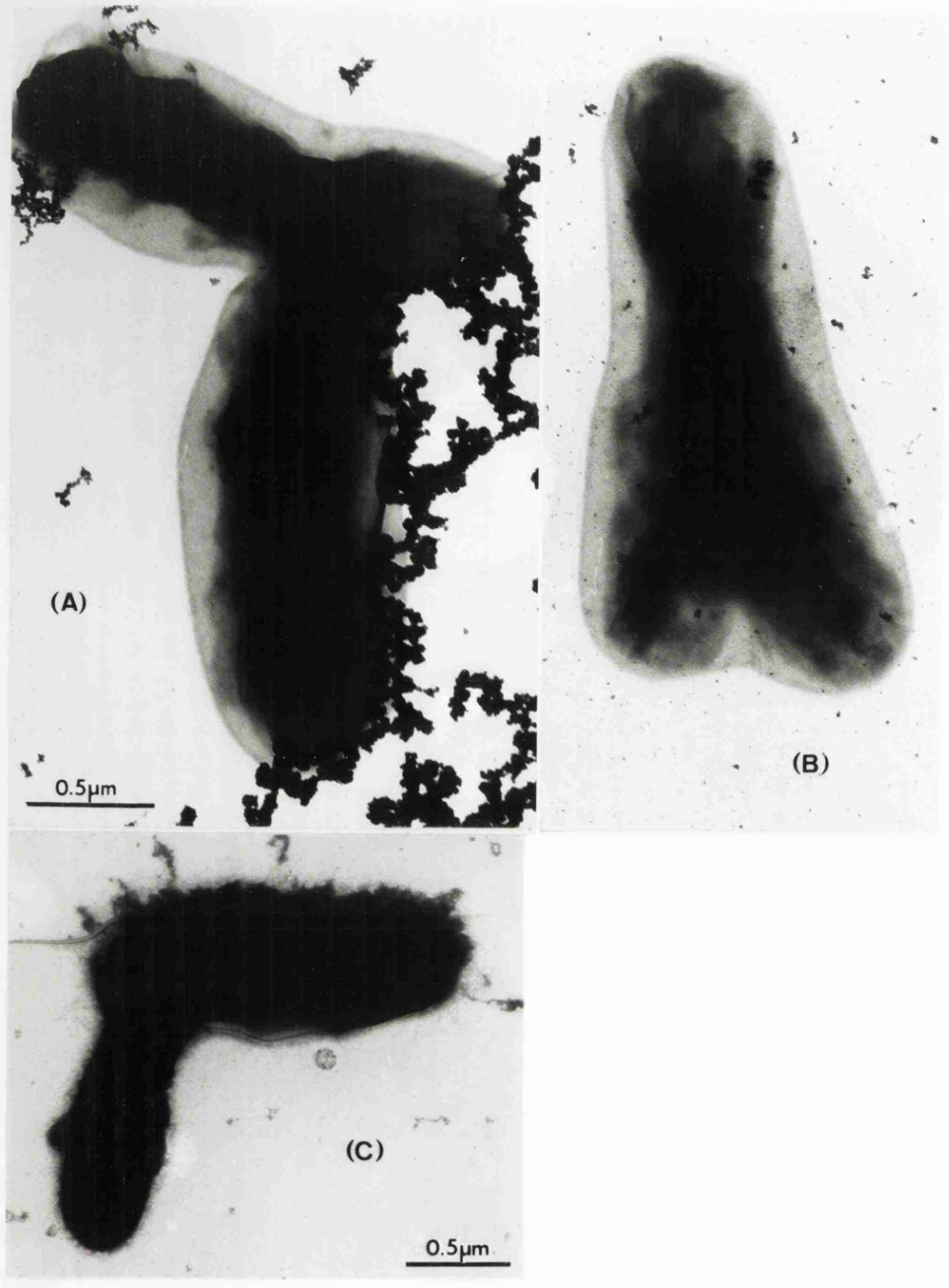


Figure 3.1. Transmission electron micrographs of *A. caulinodans* showing pleomorphic shapes and envelopes. Cells were grown in batch culture on succinate minimal salts media (as used for succinate-limited continuous culture described in Materials and Methods) at 40% DOT, pH 6.8, 30°C and harvested after 3 days. (A) T-shaped cell. (B) Y-shaped cell. (C) L- shaped cell. Preparation of samples for examination was kindly performed by Ms. S. Hill, EM services, Shell Research Ltd, Sittingbourne, Kent U.K.

bium by Dhar and Ramkishma (1987).

Kaneshiro *et al.* (1983), reported pleomorphic changes for *Rhizobium* strain 32H1 and for *B. japonicum* strains USDA 26 and 110, grown on a glutamate-mannitol-gluconate-yeast extract medium. The authors reported that the number of pleomorphic cells for *Rhizobium* strain 32H1 was coincident with acetylene-reducing activity. However, *B. japonicum* strain USDA 110 which also underwent the morphological changes failed to reduce acetylene. The pleomorphs also were shown to accumulate granular storage material similar to *A. caulinodans*. This was also a feature reported for pleomorphic cells of *R. trifolii* 0403 grown on succinate (Urban and Bechtel, 1984).

The ability of dicarboxylic acids (succinate, malate, fumarate and malonate) but not sugars (glucose, fructose and arabinose), to induce pleomorphic changes was reported for *B. japonicum* strain USDA I-110 (Reding and Lepo, 1988). The morphological changes were shown to be dependent upon the carbon substrate and its concentration, but independent of pH or Na^+ ion concentration. The authors concluded that the pleomorphic forms behaved similarily to symbiotic bacteroids, and that dicarboxylic acids may serve as physiological determinants of some aspects of the bacteroid shape and form.

Similar conclusions may be valid for *A. caulinodans* with respect to carbon source effects, however, as differentiation of *A. caulinodans* was observed in ammonium-excess cultures, a correlation between pleomorphy and nitrogen fixation is unlikely. Reduced oxygen status has been suggested as a possible inducer of pleomorphy (Pankhurst and Craig, 1978). However, the phenomenon in this study was not restricted to oxygen-limited cultures. The exact nature of the causative agent of pleomorphy and its importance with respect to nitrogen-fixation remains unclear.

3.2.3 The effect of growth conditions on respiration rates of *A. caulinodans*.

The respiratory activity (potential qO_2) of whole cells of *A. caulinodans* sampled directly from the chemostat ($D=0.10h^{-1}$), was approximately three times higher during growth under succinate limitation than under either oxygen or sulphate limitation (Table 3.2). Furthermore, in contrast to the other two limitations, it was greatly in excess of the *in situ* qO_2 of the growing culture. This result suggested that either *in situ* succinate respiration was a closely regulated process, or that the *in situ* succinate concentration was below saturation.

Endogenous respiration rates of washed cells were highest following growth under succinate-excess conditions, suggesting the presence of an internal storage product, probably PHB (Stam, 1986).

Only cells prepared from succinate-limited cultures oxidised glucose and gluconate, albeit at relatively low rates compared to cells of *R. leguminosarum* grown on glucose (Ratcliffe *et al.*, 1980), and suggested the presence of an auxiliary carbon scavenging system which was derepressed under carbon-limited conditions (Harder and Dijkhuisen, 1983).

Potential respiration rates with succinate were approximately two to three times higher in cells grown under succinate limitation than under oxygen or sulphate limitation, which suggested that either the succinate transport system of cells grown under succinate-limited conditions was strongly derepressed compared to cells grown under succinate-excess conditions (see section 6.2.4), or that a transport system with a lower Km had been synthesised. Similar rates were obtained with malate supporting the idea of a common C_4-dicarboxylate transport system. It is unlikely that Sdh activity was responsible because Sdh specific activites were higher under oxygen-limited and sulphate-limited conditions than under succinate-limited

Table 3.2 The respiratory activities of *A. caulinodans* following growth in continuous culture ($D=0.10h^{-1}$) under various nutrient limitations. Washed cells were prepared and assayed for oxygen uptake as described in Materials and Methods. The potential qO_2 was measured using a sample of culture taken directly from the chemostat. Respiration rates of washed cells were corrected for endogenous respiration and/or auto-oxidation.

Substrate	Growth-limiting nutrient		
	Succinate	Oxygen	Sulphate
	(ng-atom O min^{-1} [mg cell dry wt]$^{-1}$)		
Succinate (potential qO_2)	370 ± 19(3)	138 ± 8(3)	211 ± 8(3)
In situ qO_2	122	162	160
Endogenous	16 ± 3(3)	75 ± 5(3)	32 ± 5(3)
Glucose	94 ± 5(3)	0 (3)	0 (3)
Gluconate	67 ± 14(3)	0 (3)	0 (3)
Succinate	288 ± 8(3)	58 ± 16(3)	78 ± 7(3)
Malate	199 ± 13(3)	57 ± 19(3)	62 ± 7(3)
Ascorbate-TMPD	904 ± 20(3)	1315 ±183(3)	861 ± 11(3)

conditions.

In contrast to the oxidation of succinate and malate, the oxidation of the artificial electron donor ascorbate-TMPD (which allows respiration *via* a *c*-type cytochrome linked to a cytochrome oxidase) was significantly higher in cells grown under oxygen limitation than under either succinate or sulphate limitation.

3.2.4 The effect of growth conditions on the cytochrome content of *A caulinodans*.

Qualitative and quantitative cytochrome analysis was performed on cells of *A. caulinodans* grown under various nutrient limitations ($D = 0.10h^{-1}$).

Dithionite-reduced *minus* ferricyanide-oxidised difference spectra (and corresponding second order derivative spectra), of broken cells prepared from succinate-limited cultures showed the presence of *b*-type cytochrome (shoulder at 558nm), *c*-type cytochrome (peak at 550nm) and cytochrome aa_3 (peak at 600nm) (Fig 3.2, Table 3.3). This type of cytochrome profile has previously been reported for *A. caulinodans* (Stam *et al.*, 1984) and also for other species of *Rhizobium* (Appelby, 1969; de Hollander and Stouthamer 1980). Dithionite-reduced + carbon monoxide *minus* dithionite-reduced difference spectra confirmed the presence of cytochrome aa_3 (trough at 600nm).

Similar spectral analyses of broken cells prepared from cultures grown under either oxygen or sulphate limitation confirmed the presence of *b*- and *c*- cytochromes but showed that cytochrome aa_3 was absent from oxygen-limited cultures.

Spectral analysis of broken cells prepared from phosphate-limited cultures proved unsuccessful as the preparations were unusually pink (λ_{max} 420nm and 510nm), the colour being associated with

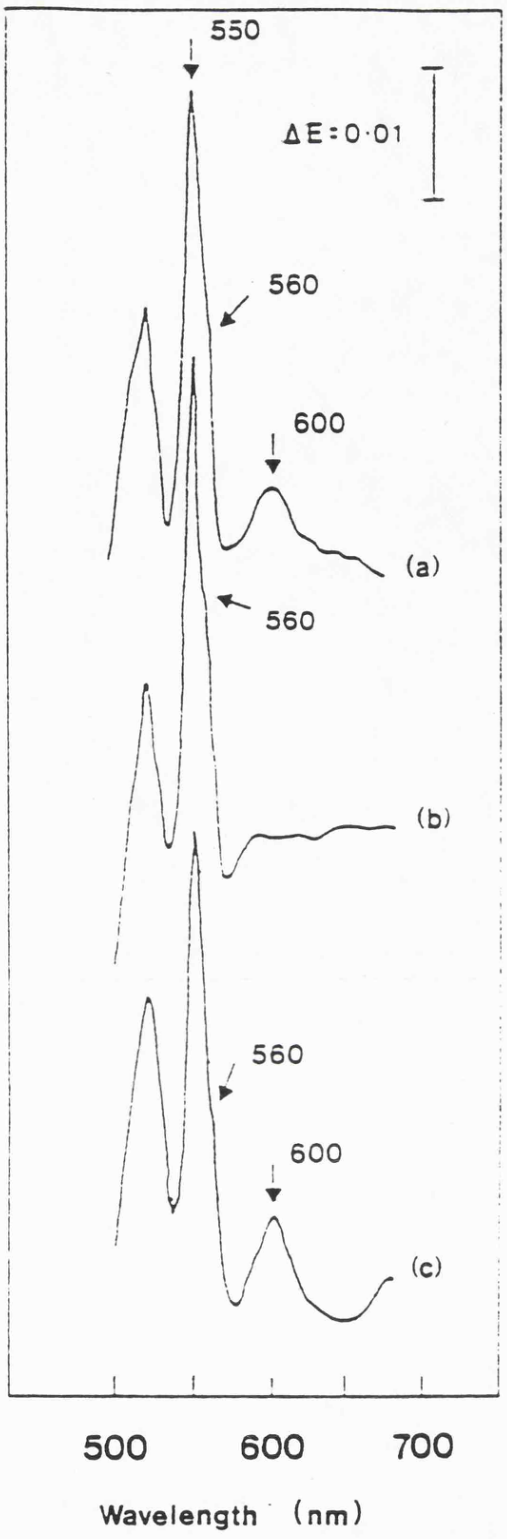


Figure 3.2. Representative dithionite-reduced *minus* ferricyanide-oxidised difference spectra of *A. caulinodans* grown under various nutrient limitations. Broken cells (6 mg dry wt ml^{-1}) were prepared as described in Materials and Methods. Nutrient limitation: (a) succinate, (b) oxygen, (c) sulphate.

Table 3.3. The cytochrome content of *A. caulinodans* following growth in continuous culture (D=0.10 h^{-1}) under various nutrient limitations. Broken cells were prepared and assayed for cytochromes as described in Materials and Methods. ND, not detected; +, from PAGE and/or inhibitor studies.

Cytochrome	Growth-limiting nutrient		
	Succinate	Oxygen	Sulphate
	(pmol [mg cell dry wt]$^{-1}$)		
Total *b*	129 ± 8(3)	138 ± 4(3)	81 ± 7(3)
Total *c*	165 ± 4(3)	202 ± 5(3)	145 ± 5(3)
aa_3	55 ± 3(3)	ND	44 ± 5(3)
co	+	++	+
d	ND	+	ND

the particulate material of the cell; broken cells remained rather cloudy even after extensive sonication and exhibited poorly defined cytochrome spectra.

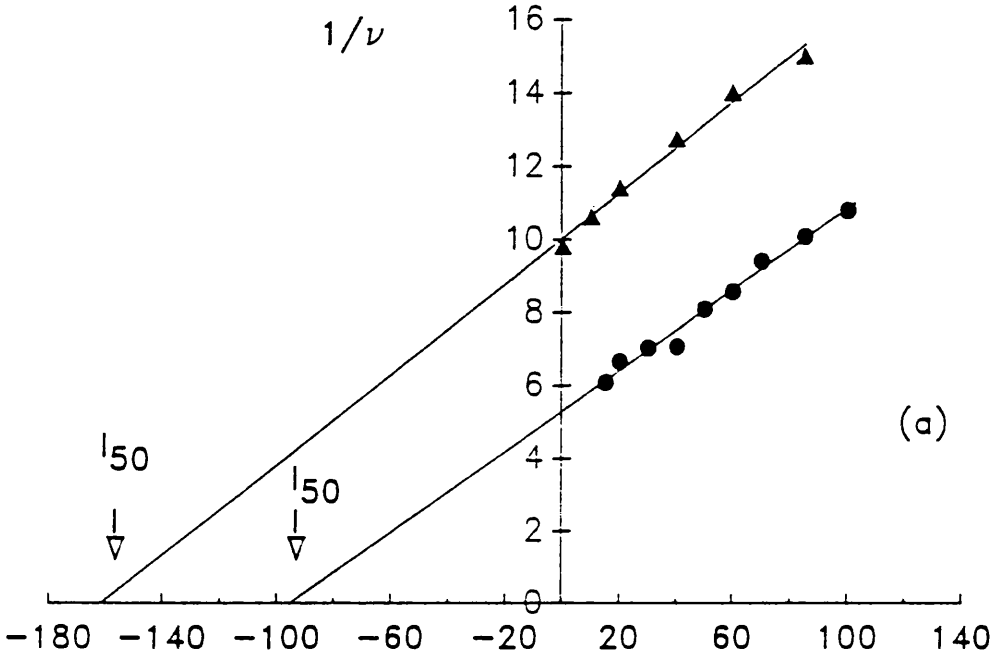
In view of the failure to detect an *o*-type cytochrome in CO-difference spectra its possible presence was investigated by subjecting broken cell samples to PAGE and then staining the gels for haem-associated peroxidase activity. Prominent haem-containing bands of 24 and 29 kDa were observed for cells grown under succinate, oxygen and phosphate limitation. These bands were commensurate with the presence of cytochrome *co* (*o* 29 kDa; *c* 24 kDa) as has been observed in several other Gram negative bacteria (King and Drews, 1976; Jurtshuk and Yang, 1980; Matsushita *et al.*, 1982; Carver and Jones, 1973; Froud and Anthony, 1984).

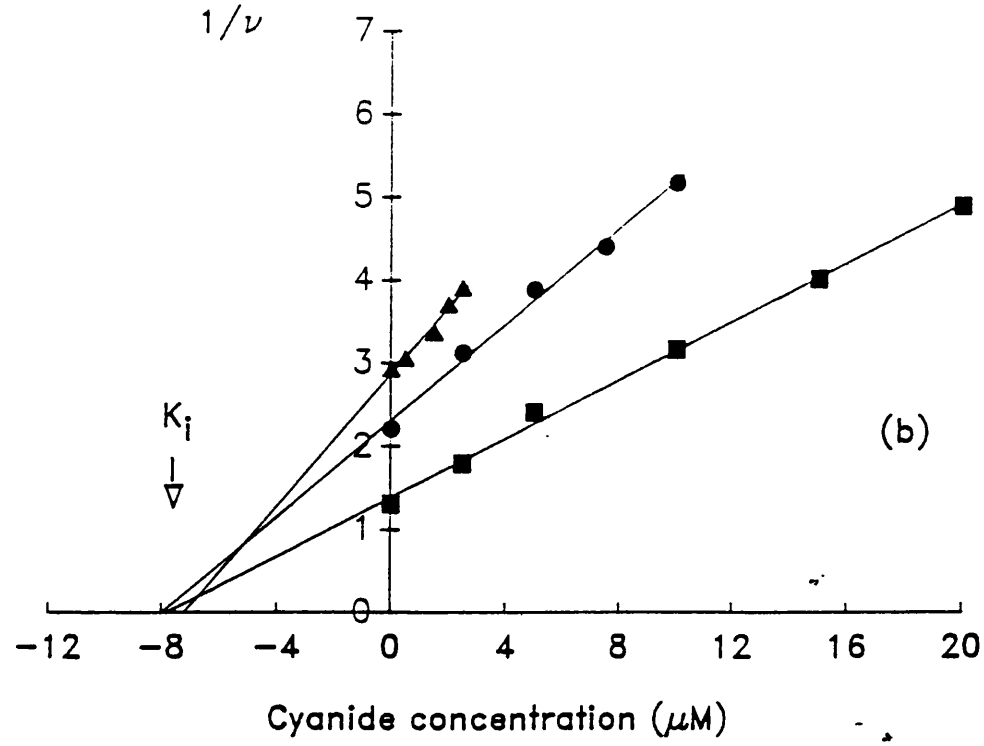
The 29 kDa protein was derepressed during growth under oxygen limitation. The haem-positive band which ran with the dye front in tracks of phosphate-limited cells was characteristic of free haem. This was further verified using a 15% polyacrylamide gel, where a strong haem-positive band was again found at the dye front.

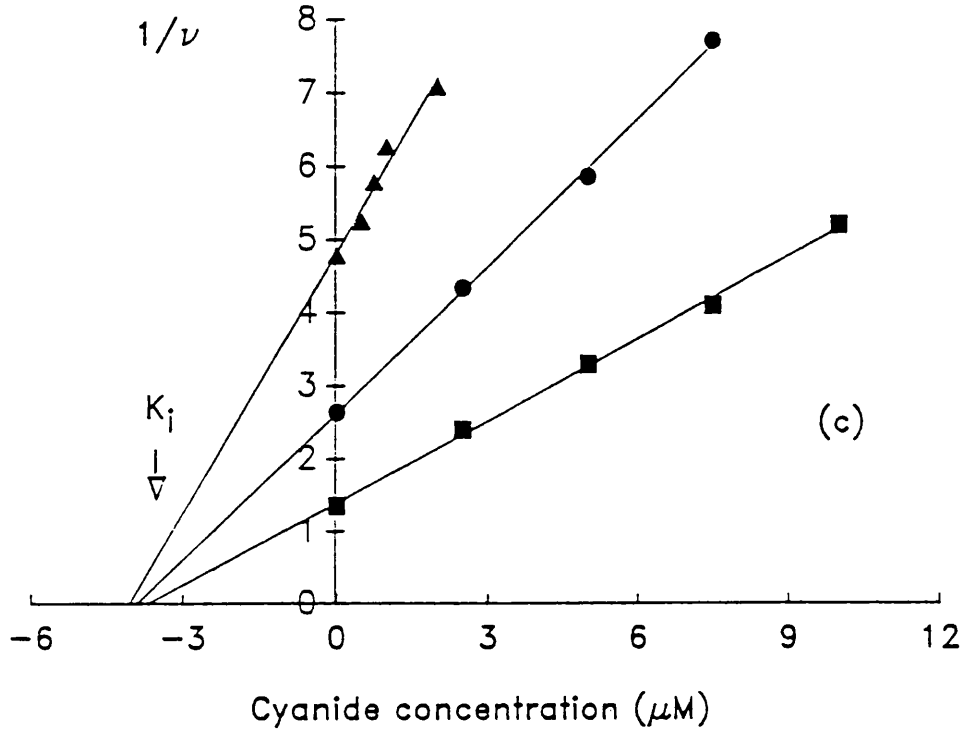
3.2.5 The effect of inhibitors on respiratory activity.

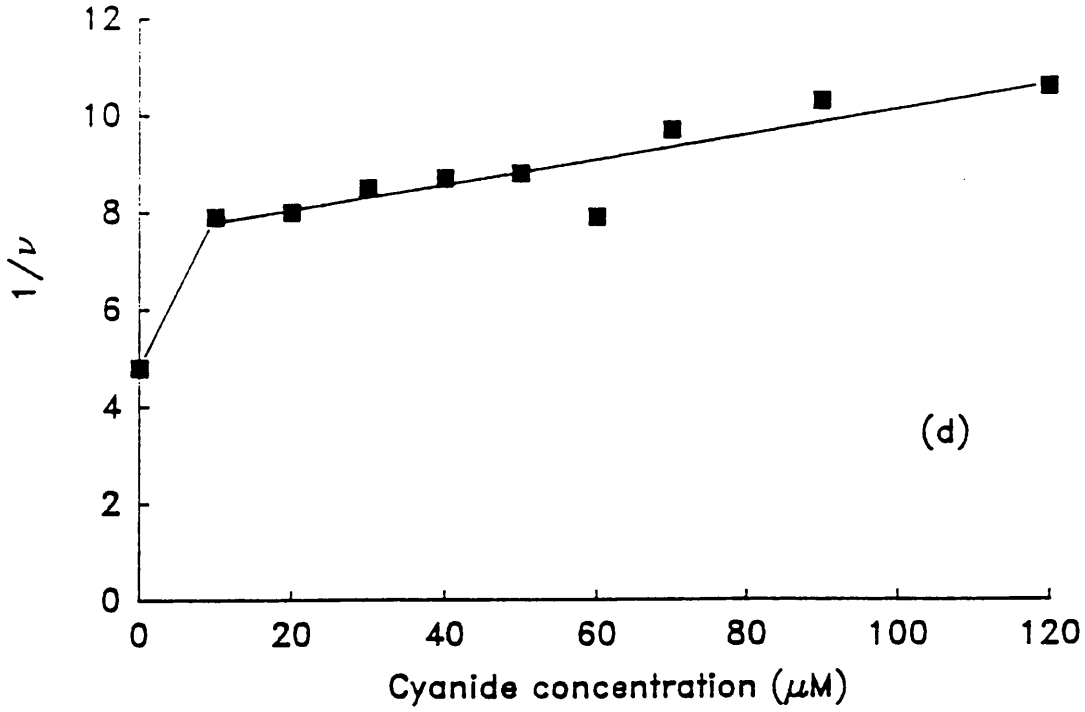
Inhibition of respiration with cyanide can yield characteristic inhibition kinetics typical of certain terminal oxidase activity. Uncompetitive inhibition was found for the oxidation of physiological substrates by cells grown under succinate and oxygen-limited conditions (Figs 3.3a, 3.3b) which was commensurate with the presence of terminal oxidases aa_3 or *d*. The I_{50} values of 94 μM for succinate oxidation and 158 μM for gluconate oxidation were considerably lower than expected for cytochrome oxidase *d* (Fig 3.3d) (see Jones 1973; Carver and Jones, 1973; Froud and Anthony, 1984; Cornish

Figure 3.3*abcd*. The effect of cyanide on the respiratory activities of washed cells of *A. caulinodans* following growth in succinate-limited (a,b) or oxygen-limited continuous culture (c,d) (D = $0.1h^{-1}$). Washed cells were prepared and assayed for oxygen uptake as described in Materials and Methods. Cells were pre-incubated with cyanide for 1 min prior to the addition of substrate, and the resultant respiration rates (v) corrected for endogenous respiration and/or auto-oxidation (v is expressed as μg-atom O min^{-1} [mg cell dry wt]$^{-1}$). (a) Succinate 1mM (●), gluconate 1mM (▲); (b), Ascorbate-TMPD: 200 μM TMPD (▲), 300 μM TMPD (●), 1mM TMPD (■). (c) Ascorbate-TMPD: 100 μM TMPD (▲), 300 μM TMPD (●), 1mM TMPD (■). (d) Ascorbate-TMPD: 1mM TMPD.


1/ν
16
14
12
10
8
6
4
2
0
I50
I50
(a)
−180 −140 −100 −60 −20 20 60 100 140
Cyanide concentration (μM)


1/ν
7
6
5
4
3
2
1
0
Ki
(b)
−12 −8 −4 0 4 8 12 16 20
Cyanide concentration (μM)


1/ν
8
7
6
5
4
3
2
1
0
Ki
I
V
(c)
−6
−3
0
3
6
9
12
Cyanide concentration (μM)


12
10
8
6
4
2
0
1/ν
(d)
0
20
40
60
80
100
120
Cyanide concentration (μM)

et al., 1987).

In contrast, noncompetitive inhibition kinetics were observed for the oxidation of ascorbate-TMPD by cells grown under succinate-limited conditions (Fig 3.3b), yielding an I_{50} ($\simeq K_i$) of 10 μM. A similar situation probably also pertained for the inhibition of succinate oxidation by low concentration of cyanide (Fig 3.3a). These low K_i values were characteristic of cytochrome oxidase *co* or *o*.

Cells grown under oxygen-limited conditions also exhibited noncompetitive inhibition kinetics with a K_i value of 4 μM for the oxidation of ascorbate-TMPD (Fig 3.3c), which was again characteristic of cytochrome oxidase *co* or *o*. However, at higher concentrations of cyanide the inhibition became uncompetitive (Fig 3.3d), with an I_{50} of approximately 300 μM, which suggested the presence of cytochrome oxidase *d*.

The results of the spectral analyses, SDS-PAGE and cyanide inhibition experiments, together with the substantially higher rates of ascorbate-TMPD oxidation in oxygen-limited cultures, suggested the presence in *A. caulinodans* of cytochrome oxidase *co* in addition to cytochrome oxidases aa_3 and possibly *d*. Cytochrome oxidase *co* is also present in several other Gram-negative aerobes including *Azotobacter vinelandii* and *Methylophilus methylotrophus* (Carver and Jones, 1973; King and Drews, 1976; Jurshuk and Yang, 1980; Matsushita *et al.*, 1982; Froud and Anthony, 1984; Cornish *et al.*, 1987). The observed cytochrome patterns under the different nutrient limitations indicated that cytochrome oxidase aa_3 was induced by excess oxygen, whereas, cytochrome oxidases *co* and *d* (if present) were partially or completely repressed by excess oxygen. Cytochrome *c* was slightly derepressed under oxygen-limited conditions, (possibly due to the increased synthesis of cytochrome *co*) whereas, cytochrome *b* expression was independent of both oxygen and carbon concentration, but appeared to be diminished by low levels of sulphate (the reason for which is unclear).

3.2.6 Determination of biomass yields with respect to succinate, oxygen and carbon dioxide during growth under succinate limitation.

Molar growth yields based on the utilization of carbon substrate or oxygen reflect the efficiency with which aerobic bacteria conserve energy *via* substrate-level and oxidative phosphorylation, and then subsequently utilise this energy for growth. In aerobes growing under carbon limitation the yields primarily reflect the efficiency of respiratory chain-linked energy conservation.

The effect of growth rate on the physiology of *A. caulinodans* was studied in succinate-limited continuous chemostat culture (D = 0.03 to $0.14h^{-1}$). The cell density was found to be linearly dependent upon the concentration of succinate present in the medium (Fig 3.4), clearly showing that the culture was succinate-limited for growth. Analysis of the effluent medium and gas showed that no products other than cells and carbon dioxide were formed. Elemental analysis of freeze-dried samples showed the cell composition corresponded to the elemental formula ($C_1H_{1.82}N_{0.19}O_{0.52}P_{0.01}$) (Table 3.4). This changed very marginally when the cells were grown at dilution rates above or below $0.10h^{-1}$.

The *in situ* rates of succinate utilization, oxygen consumption and carbon dioxide production all increased as a linear function of dilution rate when measured at various dilution rates between 0.03 and $0.14h^{-1}$ (μ_{max} = $0.16h^{-1}$) (Fig 3.5). Y^{max} values of 42.4 g dry wt (mol succinate)$^{-1}$, 27.7 g dry wt (mol oxygen)$^{-1}$ and 19.0 g dry wt (mol carbon dioxide)$^{-1}$ were calculated from the slopes since Y^{max} = 1/slope (Pirt, 1975). These growth yields were very close to those reported previously for succinate-limited cultures of *A. caulinodans* (Stam *et al.*, 1984) and *Acinetobacter calcoaceticus* (Hardy and Dawes 1985; Fewson, 1985) grown under similar conditions.

Extrapolation of the $q_{substrate}$ versus Dh^{-1} plot (Fig 3.5) yielded a maintenance energy coefficient (*M*) of zero or slightly

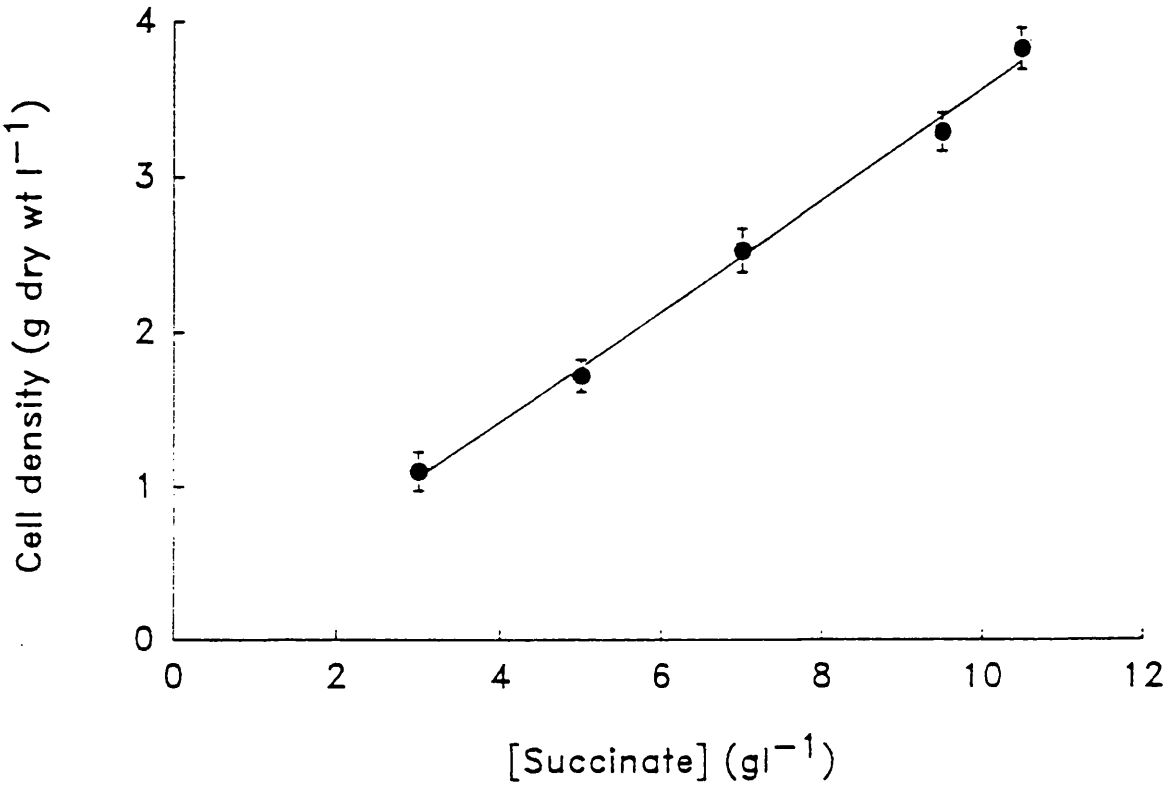


Figure 3.4. The effect of succinate concentration in the input medium on steady-state cell densities (g bacterial dry wt l^{-1}) for *A. caulinodans* grown ammonium-assimilating and succinate-limited in a chemostat at a dilution rate of $0.10h^{-1}$. The values represent the mean of at least 6 independent determinations (± SEM).

Table 3.4. Elemental analysis of *A. caulinodans* grown under succinate limitation in continuous culture at various dilution rates. Elemental analysis was carried out on freeze-dried cells as described in Materials and Methods. The percentage oxygen content was calculated by difference from the measured carbon, hydrogen, nitrogen phosphorus and assuming a cell sulphur content of 1%.

Element	Dilution rate (h^{-1})			
	0.028	0.065	0.100	0.140
	Elemental composition (%)			
Carbon	47.6	46.8	45.7	45.3
Hydrogen	7.2	7.0	6.9	7.1
Nitrogen	10.3	10.1	10.5	10.5
Oxygen	33.6	34.8	35.3	35.6
Phosphorus	1.3	1.4	1.6	1.5

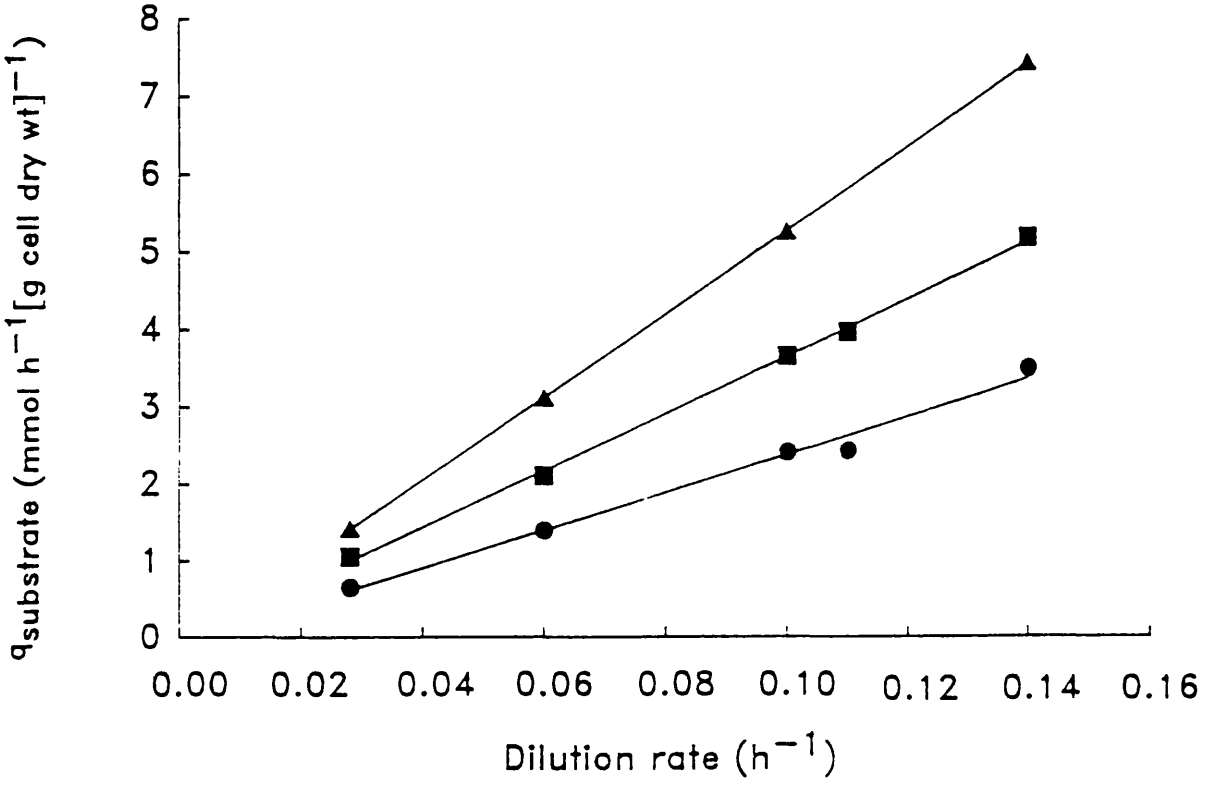


Figure 3.5. The effect of dilution rate on the rates of succinate utilisation, oxygen uptake and carbon dioxide production by *A. caulinodans* during steady-state succinate-limited growth in contiuous culture. (●), $q_{succinate}$; (■), qO_2 ; (▲), qCO_2.

negative value for *A. caulinodans* as the extrapolated lines passed through the abscissa. It is, however, likely that the (*M*) values were very low and probably (if measured correctly) of the same order of magnitude as those already reported for *Beneckea natrigens* (Linton *et al.*, 1977); *R. leguminosarum* (Ratcliffe *et al.*, 1983) and *Xanthomonas campestris* (Rye *et al.*, 1988) cultured on glucose ($M_{glucose}$ = 0.04 mmol glucose h^{-1} [g cell dry wt]$^{-1}$), but lower than the $M_{succinate}$ value of 0.67, calculated for *Acinetobacter calcoaceticus* (Fewson, 1985), cultured on succinate. The reason for the increased $M_{succinate}$ calculated for *A. calcoaceticus* compared to *A. caulinodans* is not clear but may reflect a lower level of energetic efficiency, alternatively, the higher ionic strength of the growth medium (Hardy and Dawes, 1985), compared to that used for *A. caulinodans* may have exerted an osmotic effect on the cell (see Watson, 1970; and Stouthamer & Bettenhausen, 1973).

The stoichiometry of carbon-limited growth can be cross-checked by comparison of the theoretical Yo_2 and Yco_2 values calculated from the observed $Y_{succinate}$ and the appropriate mass balance equation (see Appendix 2). The theoretcal Yo_2 values calculated for *A. calcoaceticus* (Fewson, 1985) were lower than those observed *in situ* for *A. caulinodans*, but matched very closely the calculated values (Table 3.5). Elemental analysis of freeze-dried cells for *A. calcoaceticus* (Hardy and Dawes, 1985) were also similar to this study. The calculated Yo_2 and Yco_2 values were lower than the *in situ* values. However, comparison of the respiratory quotients (ratio of the volume of carbon dioxide expired to the volume of oxygen consumed during the same time) clearly showed that the ratios of Yo_2 to Yco_2 were the same.

The difference between the calculated and the measured yields was possibly due either to the mass balance equation being over simplified, or that hydrogen was unidentified during the CHNP analysis. A cellular hydrogen content of 2.6 g-atoms mole carbon^{-1} would make the calculated values of Yo_2 and Yco_2 equal to the measured

Table 3.5. Molar growth yields of *A. caulinodans* grown at various dilution rates under succinate limitation in continuous culture, as described in Materials and Methods. Theoretical YO_2 and YCO_2 values were calculated using a modified mass balance equation of Herbert (1976), and an empirical cell formula of $CH_{1.82}N_{0.19}O_{0.56}P_{0.01}$, derived from cell elemental analysis. Theoretical $Y_{succinate}$ values were determined by calculation from the *in situ* YO_2 and YCO_2. (n = number of determinations).

	Dilution rate (h^{-1})				
	0.028	0.065	0.100	0.110	0.140
$Y_{succinate}$ (g cell mol^{-1})	42.24	41.94	41.46	44.76	40.05
Y_{oxygen} (g cell mol^{-1})	26.20	28.10	27.30	27.50	27.00
$Y_{carbon\ dioxide}$ (g cell mol^{-1})	19.59	18.83	19.01	24.26	18.81
Carbon recovery (%)	100.83 (n=11)	99.70 (n=12)	102.00 (n=12)	91.80 (n=8)	95.50 (n=10)
Respiratory quotient (RQ)	1.33	1.49	1.43	1.11	1.43
Theoretical Molar growth Yields					
$Y_{succinate}$	41.27	36.31	36.75	39.74	36.07
Y_{oxygen}	23.30	23.00	22.50	26.14	21.10
$Y_{carbon\ dioxide}$	17.90	17.70	17.33	19,77	16.40
RQ	1.30	1.30	1.30	1.31	1.28

values.

Preliminary measurements of $\rightarrow H^+/O$ quotients for the oxidation of endogenous substrates using SCN^- as the $\Delta \Psi$ collapsing agent, suggested a value of 6 with a $t_{\frac{1}{2}}$ (time taken for the Δ pH to decrease by 50%) of approximately 12s (Fig 3.6). This result was in close agreement with the value reported by Stam *et al.* (1984).

Both the $\rightarrow H^+/O$ quotient of 6 and the $t_{\frac{1}{2}}$ value of 12s were similar to those previously described for *R. leguminosarum* (Ratcliffe *et al.*, 1983) and *Xanthomonas campestris* (Rye *et al.*, 1988). This rapid rate of decay of the pH gradient generated by respiration was at least three times faster than has been observed for many other species of mesophilic bacteria under comparable conditions ($t_{\frac{1}{2}}$ > 40s) (Rye *et al.*, 1988), and may reflect energy dissipation, which in turn could be partly responsible for the low growth yields exhibited by these organisms.

The theoretical value for Y^{max}_{ATP} was calculated from the method of Stouthamer (1979), and an elemental formula for *A. caulinodans* corresponding to a molecular weight of 100 (see Table 3.6), was used to construct an equation for assimilation from succinate:

$$C_4H_6O_4 + 0.76NH_3 + 1.42H_2 \text{ (as } NADH_2) \rightarrow C_4H_{7.28}O_{2.08}N_{0.76} + 1.92H_2O$$

The consumption of $1.42H_2$ was included in the equation where "H_2" indicated the requirement for reducing equivalents in the formation of biomass.

Calculation of the $Y^{max}_{succinate}$ using the Y^{max}_{ATP} value of 15.4 from Stouthamer (1979), generated values for $Y^{max}_{succinate}$ of 43.17, 58.41 and 67.22 for P/O quotients of 1, 2 and 3 respectively.

Oxygen-limited and sulphate-limited cultures of *A. caulinodans* ($D = 0.10h^{-1}$, pH 6.8) exhibited substantially higher rates of succinate utilization, oxygen consumption and carbon dioxide production than succinate-limited cultures, and hence significantly lower molar growth yields (Table 3.7).

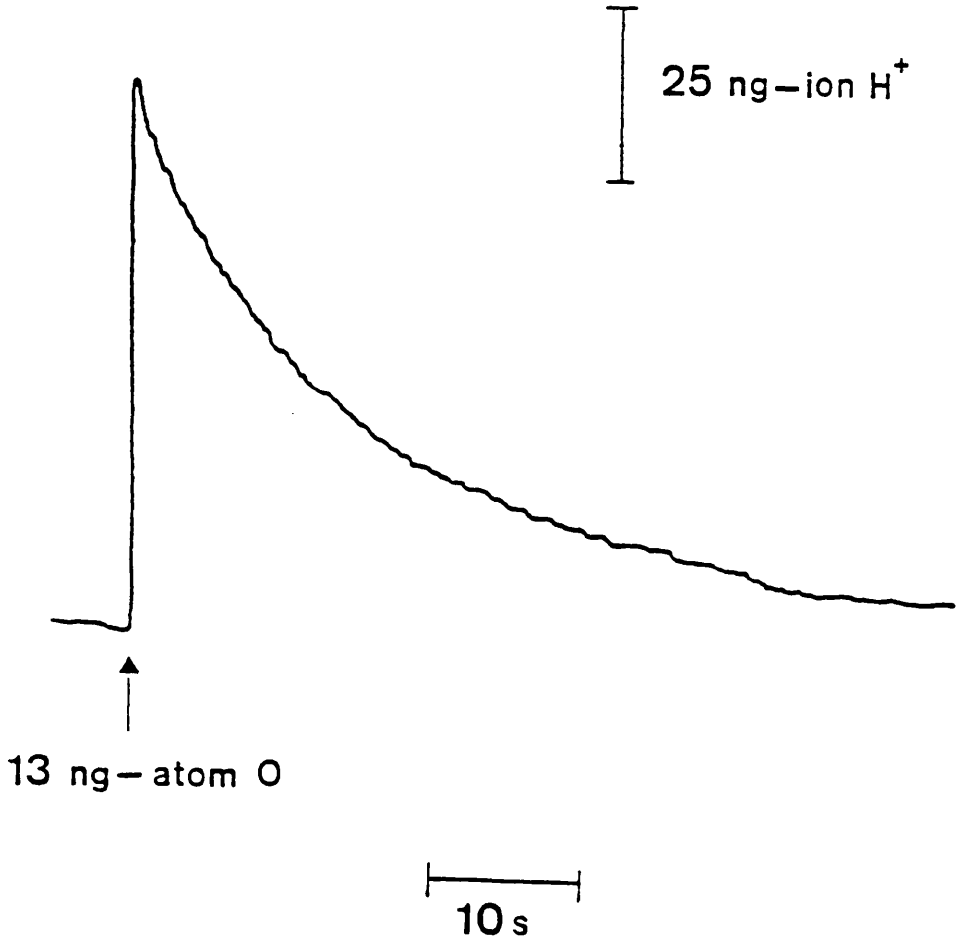


Figure 3.6. Typical pH response to an oxygen pulse for anaerobic *A. caulinodans*. $\rightarrow H^+/O$ quotients were determined for the oxidation of endogenous substrate in the presence of SCN^- (50 mM), according to the method of Mitchell and Moyle (1967).

Table 3.6. Calculation of theoretical maximum growth yields on succinate and oxygen.

Assuming that the complete dissimilation of 1 mol succinate yields 5 mol $NADH_2$, 2 mol $FADH_2$ and 1 mol ATP (from substrate level phosphorylation), equations for the amount of succinate assimilated and dissimilated, and the reducing power available for oxidation can be constructed: where the molecular weight of the cell = 100, succinate assimilated = $Y^{max}_{succinate}$ /100, succinate dissimilated = 1 - ($Y^{max}_{succinate}$ /100); $NADH_2$ required for assimilation = 1.42 x $Y^{max}_{succinate}$ /100; reducing equivalents available for oxidation = 5 - (5 x $Y^{max}_{succinate}$) - (1.42 x $Y^{max}_{succinate}$) = (5 - 6.42$Y^{max}_{succinate}$)$NADH_2$, and 2 - (2 x $Y^{max}_{succinate}$/100)$FADH_2$. The total ATP gain from the utilisation of 1 mol succinate is described by the equations derived on the basis of one, two or three phosphorylation sites. Since $Y^{max}_{succinate}$ = (Y^{max}_{ATP}) x (total ATP per mol succinate), each value of Y^{max}_{ATP} can be calculated (Stouthamer, 1979).

Table 3.6. Theoretical calculations of maximum growth yields on succinate and oxygen.

P/O quotient	1	2	3
Source of ATP:			
NADH	5-(6.42Y/100)	10-(12.84Y/100)	15-(19.26Y/100)
$FADH_2$		2-(2Y/100)	4-(4Y/100)
Substrate-level phosphorylation	1-(Y/100)	1-(Y/100)	1-(Y/100)
Total ATP	6-(7.42Y/100)	13-(15.84Y/100)	20-(23.26Y/100)
Y^{max}_{ATP} =	12.7	6.65	4.12
From Y_{oxygen}[a]	13.9	6.93	4.62

Where $Y = Y^{max}_{succinate}$

[a] = The Y^{max}_{ATP} calculated from the Y^{max}_{oxygen} using the equation of Pirt (1975):

$$Y^{max}_{oxygen} = Y^{max}_{ATP} \times (2 \times P/O)$$

Table 3.7. The effect of nutrient limitation on the observed molar growth yields of *A. caulinodans* ($D = 0.1h^{-1}$). Molar growth yields were measured as described in Materials and Methods.

Nutrient-limitation	Steady-state [succinate] (mM)	Y_{succ} (g cell dry wt mol^{-1})	Yo_2 (g cell dry wt mol^{-1})	Yco_2 (g cell dry wt mol^{-1})	Carbon recovery (%)
Succinate	~0	41.5	27.3	19.0	102.0
Oxygen	27.1	39.8	20.6	17.2	96.3
	64.6	38.0	17.1	14.9	96.3
	112.7	34.5	14.0	12.0	98.3
	190.7	30.4	12.0	13.0	94.0
Sulphate	107.5	39.0	20.8	17.0	97.2

The respiratory quotients were slightly higher in oxygen-limited cells than for sulphate limitation (1.16 and 1.22 respectively), at the equivalent inflowing medium succinate concentration ($\simeq$ 115 mM). No extracellular products other than cells, carbon dioxide and unmetabolised succinate were detected, but under both limitations the cells contained substantial amounts of intracellular poly-ß-hydroxybutyrate (up to 0.37 g g cells^{-1}) which was independent of the steady-state concentration of succinate in the medium. The PHB was probably formed to dispose of excess carbon and reducing power during growth under succinate-excess conditions (Stam *et al.*, 1984; de Vries *et al.*, 1984). The molar growth yields for oxygen and sulphate-limited cultures were not corrected for PHB accumulation.

The molar growth yields of oxygen-limited cultures varied inversely with the steady-state concentration of succinate in the growth medium; this phenomenon was investigated further (see chapter 4).

The Y_{oxygen} and $Y_{succinate}$ values were similar to those of Stam (1986) for *A. caulinodans*, at a steady-state succinate concentration of approximately 100 mM, and to that of 16.6 and 42.7 g mol^{-1} respectively for *A. calcoaceticus* by Hardy and Dawes (1985). Unfortunately, little other comparative data exists on the molar growth yields of oxygen or sulphate-limited bacterial cultures using succinate as a carbon source.

3.2.7 Effect of nutrient limitation and growth rate on enzyme actvities.

Succinate dehydrogenase, malate dehydrogenase and NADH oxidase activities were measured in broken cells prepared from succinate,

oxygen and sulphate-limited cultures (D = $0.10h^{-1}$). Succinate dehydrogenase activity was highest in oxygen and sulphate-limited cells (Table 3.8). Malate dehydrogenase activity was highest in oxygen-limited cells (followed by sulphate-limited cells) and NADH oxidase activity was highest in oxygen-limited cells.

Succinate dehydrogenase, malate dehydrogenase and NADH oxidase activity all increased with increasing growth rate in succinate-limited cells, with the exception of D=0.03 h^{-1} for the latter two (Table 3.8).

The prominent protein bands (M_r = 37,000 and 39,500), from SDS-PAGE analysis of succinate-limited cells, did not correlate with the known subunits M_r of succinate dehydrogenase from *B. subtilis*, large subunit M_r = 60,000-79,000 ; small subunit M_r = 25,000-31,000. It is unlikely that these protein bands were associated with rhizobial succinate dehydrogenase since the enzyme complex is of similar composition in most bacterial spp.

Waters *et al.* (1985) reported that SDS-PAGE analysis of bacteroid malate dehydrogenase resulted in a single protein band of M_r = 36,000. The SDS-PAGE protein band of M_r = 37,000 of *A caulinodans* could perhaps associated with malate dehydrogenase, or alternatively, one of the protein bands may perhaps be associated with the C_4-dicarboxylate transport system since they were sensitive to osmotic shock (Fig 3.7) and were in the molecular weight range predicted for the transporter (Ronson *et al.*, 1984).

Table 3.8. The effect of nutrient limitation and dilution rate on succinate dehydrogenase, malate dehydrogenase and NADH oxidase activites in broken cells of *A. caulinodans*. Enzyme activities were measured as described in Materials and Methods. The results are expressed as the mean ± SEM from a minimum of 3 independent determinations.

Nutrient limitation	D (h^{-1})	Succinate dehydrogenase	Malate dehydrogenase	NADH oxidase
		(nmol min^{-1} [mg cell dry wt]$^{-1}$)		
Succinate	0.030	22.5 ± 0.5	1441 ± 81	51.4 ± 1.9
	0.065	28.0 ± 2.1	1291 ± 99	40.2 ± 6.4
	0.010	27.9 ± 1.2	1441 ± 45	52.6 ± 16.7
	0.140	33.9 ± 1.1	1918 ± 37	59.9 ± 7.7
Oxygen	0.010	38.4 ± 2.8	5031 ± 74	212.9 ± 27.6
Sulphate	0.010	43.7 ± 5.5	2701 ± 126	54.2 ± 8.3

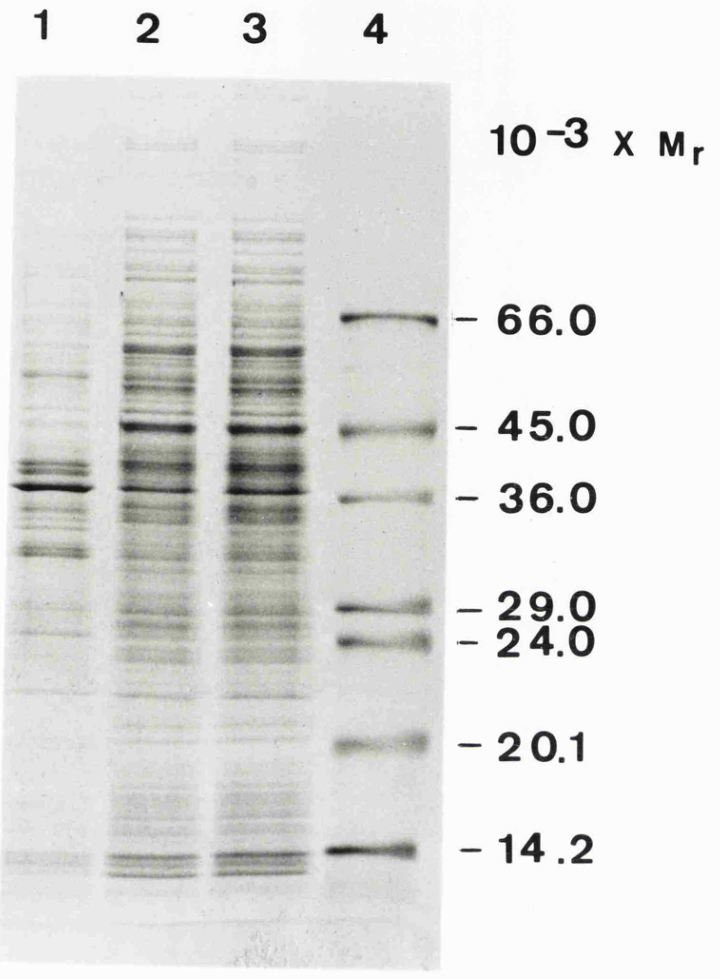


Figure 3.7. SDS-PAGE profiles of kenacid stained proteins on a 7-20% gradient gel, from osmotically shocked cells of *A. caulinodans* (as described in Materials and Methods), grown in chemostat culture under succinate limitation (D= $0.1h^{-1}$, pH 6.8). Lane 1, periplasmic shock fluid; lane 2, osmotically shocked cells; lane 3, un-shocked cells; lane 4, M_r markers.

3.3 Discussion

The range of carbon substrates utilised by *A. caulinodans* (principally organic acids), resembled closely those believed to be important in bacteroid carbon metabolism, and further highlighted the similarity between free-living *A. caulinodans* and bacteroids of other rhizobia. Organic acids are considered the primary energy source for bacteroids (Tuzimura and Meguro, 1960; Ronson and Primrose, 1979; Ronson *et al.*, 1981), supporting the highest respiration rates (Ratcliffe *et al.*, 1980; Peterson and LaRue, 1981) and hence probably the most effective substrates for supporting nitrogen fixation (see Bergersen, 1977).

The pleomorphic shapes observed when *A. caulinodans* was grown on succinate were very similar to those already described for cowpea-type *Rhizobium* strain 32H1 and *B. japonicum* strain USDA 26 and 110 (Kaneshiro *et al.*, 1983) *R. trifolii* strain 0403 (Urban and Bechtel, 1984) and *B. japonicum* strain USDA 1-110 (Reding and Lepo, 1988). The structures from *A. caulinodans* outwardly resembled bacteroids, and as reported for the above studies succinate might be an *in vitro* inducer.

The results obtained with *A. caulinodans* paralleled closely those for cowpea-type *Rhizobium* and *B. japonicum* strain USDA 26 (Kaneshiro *et al.*, 1983), with the exception that *A. caulinodans* cells were probably repressed for nitrogenase activity due to the high levels of supernatant ammonia.

Organic acids such as succinate, malate, lactate and gluconate were the preferred carbon substrates for growth of *A. caulinodans*. Some strains will grow on glucose (Dreyfus *et al.*, 1988). Glucose metabolism may have resulted from a loss of the cofactor PQQ from glucose dehydrogenase (Linton *et al.*, 1986) and hence a failure to generate gluconate. However, preliminary experiments showed that PQQ did not stimulate growth on glucose.

The importance of hexose metabolism in bacteroids is still in question; reports exist of bacteroid preparations of *B. japonicum* from soybean nodules which had an energy-dependent glucose uptake system, which were shown to phosphorylate the transported glucose; (uptake was only partially inhibited by 2-deoxyglucose suggesting two mechanisms were operative) (Jacobsen and San Francisco, 1985). However, bacteroids of *R. leguminosarum* isolated from pea nodules were incapable of transporting glucose (Hudman and Glenn, 1980; de Vries *et al.*, 1982), or even of oxidising it (Glenn and Dilworth, 1981) despite containing many of the required enzymes.

Expression of succinate dehydrogenase and malate dehydrogenase was apparently regulated by the availability of succinate, with maximum activities being observed in cells grown under excess succinate conditions. This was confirmed by showing that these activities in succinate-limited cultures increased with increasing dilution rate i.e. in response to the increasing steady-state concentration of succinate (Monod, 1942), and *in vivo* $q_{succinate}$ values. Since succinate dehydrogenase expression was controlled closely by the carbon status of the organism, it is possible that the enzyme possesses a high flux control coefficient (Kascer and Burns, 1973; Heinrich and Rapoport, 1974), and consequently is important in the regulation of succinate metabolism in *A. caulinodans*.

The high levels of malate dehydrogenase observed for *A. caulinodans* are similar to those in *B. japonicum* (Waters *et al.*, 1985; Emerich *et al.*, 1988; Copeland *et al.*, 1989;). There are few reports on the biochemistry of individual enzymes of organic acid metabolism in rhizobia. However, Salminen and Streeter (1987) reported that succinate and malate had different metabolic fates in bacteroids. Succinate was more readily converted into amino acids and used for bacteroid maintenance, whereas, malate was more readily converted to CO_2, and used as an energy source. An alternative explanation for high malate dehydrogenase expression was proposed by LaRue *et al.* (1984), who suggested that bacteroids operate a semi-fermentative

metabolism and that reversed malate dehydrogenase activity (reducing oxaloacetate) was involved in re-oxidising NADH, since aerobic respiration was limited. It is difficult to reconcile either of these two explanations with free-living *A. caulinodans*. However, patterns of malate dehydrogenase expression, for example, derepression of activity under oxygen limitation and succinate-excess conditions, are consistent with both hypotheses.

Considerable differences have been shown to exist in activities of the enzymes involved in organic acid metabolism between bacteroids of different *Rhizobium* spp. (Kurz and LaRue, 1975; Karr *et al.*, 1984), and also between bacteroids and the free-living forms (McKay *et al.*, 1988).

PHB production in *A. caulinodans* (formed during conditions of carbon-excess and oxygen limitation), may have been to act as a sink for excess reducing power when the capacity of the respiratory chain to oxidise NADH became limiting (Senior and Dawes, 1971). In bacteroids the polymer may be utilised to support nitrogen fixation during darkness or when photosynthate is not readily available (Bergersen, 1977), although this was disputed by Wong and Evans (1971) and Stam (1986).

A. caulinodans synthesised a membrane-bound respiratory chain which catalysed the oxidation of succinate and NADH, and contained *b*- and *c*-type cytochromes plus cytochrome oxidases aa_3, (*d?*) and *o*-type (probably *co*).

Cytochrome oxidases *co* and *d* were partially or completely repressed by excess oxygen, and cytochrome oxidase aa_3 was induced by excess oxygen. These changes enabled the organism to achieve the *in situ* rates of carbon metabolism and respiratory chain-linked energy chain conservation demanded by the the imposed growth rate, during growth under different nutrient limitations.

Organisms which route the majority of their terminal electron flow *via* a high-potential membrane-bound cytochrome $c \rightarrow aa_3$, *o* (*co*) system generally exhibit molar growth yields which are approximately

50% higher than those of organisms that are deficient in this system (Jones *et al.*, 1977). The presence of transhydrogenase activity (site 0) does not appear to effect growth yields and energy conservation (Jones *et al.*, 1977), and although not examined in this study, has been reported to be present in *A. caulinodans* (Stam, 1986; and Stouthamer *et al.*, 1988).

Cytochrome profiles of *A. calcoaceticus* containing *b* and *o* (no cytochrome *c*), and $\rightarrow H^+/O$ quotients of approximately 4 for the oxidation of endogenous substrates (following growth on succinate and lactate) (Jones *et al.*, 1977; Hardy and Dawes, 1985), indicated the presence of only two proton translocation sites between NADH and oxygen (sites 1 and 2). However, the molar growth yields observed on succinate (Hardy and Dawes, 1985; Fewson, 1985), suggested an ATP/O quotient of 1, i.e. close to the expected value.

Stam *et al.* (1984) also reported $\rightarrow H^+/O$ quotients of 6 for endogenous respiration in *A. caulinodans* following growth on succinate. Stam *et al.* (1984), calculated Y_{ATP} values of 6.15 and 4.63 g mol^{-1} for ATP/O quotients of 2 and 3 respectively. The results presented in this study are in close agreement with those of Stam *et al.* (1984), i.e. P/O quotient of 2, although the cytochrome analyses suggest a maximum P/O quotient of 3.

The lower than expected molar growth yields observed for *A. caulinodans* may have resulted from H^+ leakage across the coupling membrane (Ratcliffe *et al.*, 1983, Rye *et al.*, 1988). Ratcliffe *et al.* (1983), reported $\rightarrow H^+/O$ quotients of 6 for the oxidation of endgenous substrate (namely NADH) in *R. leguminosarum*. This was commensurate with the presence of cytochromes *b*, *c*, aa_3 and suggested an ATP/O quotient of 2 ($\rightarrow H^+/ATP = 3$) or 3 ($\rightarrow H^+/ATP = 2$). However, the growth yields were extremely low and were commensurate with an ATP/O quotient of only 1, possibly due to very rapid H^+ leakage across the membrane, the operation of a proton symport/antiport, or a high $\rightarrow H^+/ATP$ quotient.

Failure to make use of all the potential synthesis of ATP would

be consistent with the low observed growth yields, redox chain composition, and $\rightarrow H^+/O$ quotients of *A. caulinodans*, resulting in a low calculated Y_{ATP} value of 6.8.

CHAPTER 4

The effect of succinate on the growth of *A. caulinodans*

4.1 Introduction

A transmembrane proton gradient plays a central role in cellular energy transduction, linking the processes of respiration, ATP synthesis and some types of active transport. The chemiosmotic theory of Mitchell (1961) (see also Hooper and DiSpirito, 1985) provides an explanation of the mechanism of uncoupling agents, which are proposed to act as protonophores and hence dissipate the proton motive force (Δp). Extremely good correlations have been observed between uncoupler potency *in vivo* and protonophoric activity *in vitro* (McLaughlin and Dilger, 1980).

For many years it has been known that organic acids in their undissociated form can diffuse through the hydrophobic region of the cytoplasmic membrane (Ko and Edwards, 1975; Kell *et al.*, 1981), and act as uncouplers of oxidative phosphorylation, inhibitors of amino acid transport (Freese *et al.*, 1973; Sheu *et al.*, 1975) and inducers of membrane fusion (Kantor and Prestegard, 1975; Maggio and Lucy, 1975). These activities were attributed to their ability to interfere with the establishment and maintenance of a functional pH gradient across the membrane (Finean *et al.*, 1984). The oxidation of iron and sulphur by *Thiobacillus ferroxidans* was inhibited by acetate, pyruvate, oxalate, lactate, butyrate and fumarate, and that the effects were all pH related, decreasing pH causing increased toxicity (Tuttle and Dugan 1976).

The concentration of undissociated acid in solution depends critically on the pH and the pK_a (negative logarithm of the dissociation constant K_a) of the acid, according to the Henderson-Hasselbach equation (see Appendix 3).

The uncoupling effect of organic acids is a result of the passive diffusion of the undissociated acid into the cytoplasm (down its concentration gradient) and subsequent ionization (at the higher cytoplasmic pH value). In the absence of metabolic activity the net effect of the dissociation would be to equalise the intra- and extracellular pH values and so eliminate the Δ pH component of the proton motive force. However, as with artificial protonophorous uncouplers e.g. 2,4-dinitrophenol (DNP), many organisms can counter this effect by increasing their respiration rate , and consequently the rate at which they pump protons from the cytoplasm (Hueting and Tempest, 1977). The addition of 2,4-DNP to a glucose-limited culture of *K. aerogenes* greatly increased the maintenance energy requirements and hence decreased the growth yield (Neijssel, 1977).

The intracellular pH of many plant cells, including bacteroid-containing root nodule cells, is approximately pH 5.5-6.0 (Lyalin and Kitrova, 1976; R. Nelson *pers comm*). Organic acids, principally succinate, are believed to be the *in vivo* carbon source utilized for nitrogen fixation and maintenance of bacteroids (see 1.7.2 and 6.1). It is therefore possible that uncoupling of bacteroid respiration by succinic acid and potential acidification of the cytoplasm would also lead to increased maintenance requirements, and through the active expulsion of protons consume ATP that could otherwise be used for nitrogen fixation.

The importance of organic acids as carbon sources for nitrogen-fixing bacteroids is well documented, but their possible inhibitory effects as outlined above has not yet been addressed. In this chapter evidence is presented that the specific growth rate and the molar growth yields of *A. caulinodans* were decreased by high extracellular succinate concentrations, and that all these effects were

influenced by the culture pH.

4.2 Results

4.2.1 The effect of culture pH and potassium ion concentration on the growth rate of *A. caulinodans*.

The maximum specific growth rate of *A. caulinodans* in shake flask culture was found to be dependent on the concentration of free succinic acid and K^+ in the growth medium. (Table 4.1). A μmax of 0.155 h^{-1} was observed on standard medium (pH 6.8) containing 34 mM succinate (5 μM undissociated succinic acid) and 62 mM K^+ ion (derived from KOH neutralisation of the succinic acid; see Appendix 3), but the growth rate decreased substantially as the undissociated succinic acid and K^+ concentration were increased in parallel (i.e from 5 μM to 29 μM and 62 mM to 386 mM respectively).

A moderately low growth rate was also obtained when the medium contained 5μM undissociated succinic acid and 386 mM K^+, indicating that the diminishing growth rate at pH 6.8 was due predominantly to the effect of K^+. In contrast, when the experiment was repeated at pH 5.7 (such that the undissociated succinic acid and K^+ concentration increased from 434 to 2681 μM and from 62 mM to 386 mM respectively) growth was completely abolished, and there was a clear effect of undissociated succinic acid over and above that of K^+.

Increasing extracellular concentrations of organic acids (acetic, propionic, butyric and lactic) have also been shown to de crease the maximum specific growth rate of *Clostridium thermocellum* (Herrero *et al.*, 1985).

The effects of culture pH *per se* on the physiology of

Table 4.1. The effect of succinic acid and K^+ concentrations on the maximum specific growth rate of *A. caulinodans*. The organism was grown in batch culture at pH 6.8 and 5.7 (30°C) with potassium succinate as the carbon source as described in Materials and Methods. The K^+ concentration was adjusted as required using potassium dihydrogen phosphate, which also acted as the buffering agent. The growth rates are the mean value of three independent determinations (100% represents a μmax value of 0.155 h^{-1}). The undissociated succinic acid concentration [H_2A] was calculated from the total succinate concentration as described in Appendix 3.

Concentration in growth medium.				
Succinate (mM)	34	118	210	34
K^+ (mM)	62	216	386	386
Succinic acid [H_2A] μM				
pH 6.8	5	16	29	5
pH 5.7	434	1506	2681	434
Growth rate (% control)				
pH 6.8	100	91	58	58
pH 5.7	67	55	0	46

A. caulinodans (as distinct from the effect of undissociated succinic acid and K^+), were investigated further using succinate-limited continuous culture (D = 0.10 h^{-1}, 30°C) at pH 6.8 and 5.7. The Ysuccinate, Yo_2 and Yco_2 values respectively, were approximately 12%, 40% and 31% lower at pH 5.7 compared to 6.8 (Table 4.2).

During growth in oxygen-limited continuous culture the Ysuccinate, Yo_2 and Yco_2 values were 25%, 53% and 44% lower at pH 5.7 (43 mM K^+) than at pH 6.8 (53 mM K^+), i.e. significantly larger decreases than observed with succinate-limited cultures.

When the concentrations of undissociated succinic acid and K^+ were increased at pH 6.8 from 3.8 to 26.5 μM (Fig 4.1) and from 53 to 401 mM (Fig 4.2) respectively, the qO_2 also increased. Further increases in qO_2 were observed in pH 5.7 cultures where the undissociated succinic acid (Fig 4.1) and K^+ (Fig 4.2) concentrations were increased from 357 to 1190 μM and 43 to 140 mM respectively. Decreases in molar growth yields were observed in both cases (Table 4.2). Culture washout was observed at undissociated succinic acid concentrations above 1266 μM.

It was unlikely that the decrease in growth efficiency in the pH 6.8 cultures was a function of the undissociated succinic acid, since the concentration was very low, but was almost certainly attributable to the K^+ ion concentration, as shown by the inhibition of growth rate at pH 6.8.

The effects of undissociated succinic acid on the growth efficiency of *A. caulinodans*, as distinct from K^+ effects, was investigated further. Cells were grown in continuous culture under oxygen limitation (D = 0.03 h^{-1}), at pH 6.8 and 5.7, with the concentration of undissociated succinic acid ranging from 7 to 1231 μM with the K^+ concentration maintained at 200 mM (Table 4.3). The K^+ concentration measured using plasma-emission spectrometry matched closely the theoretical K^+ concentration.

The decrease in molar growth yields observed in the pH 5.7 cultures clearly showed the effect of undissociated succinic acid. The Ysuccinate, Yo_2 and Yco_2 values decreased by 25%, 35% and 26%

Table 4.2. The effect of nutrient limitation and culture pH on the molar growth yields of *A. caulinodans* grown in continuous culture (D = 0.10 h^{-1}, 30°C) as described in Materials and Methods.

Nutrient-limitation		Steady-state concentration			Molar Growth Yields			Carbon recovery
	pH	$[H_2A]$ (μM)	Succinate (mM)	K^+ (mM)	Ysucc (g cell dry wt mol^{-1})	Yo_2	Yco_2	(%)
Succinate	6.8	~0	~0	22	41.5	27.3	19.0	102.0
Succinate	5.7	~0	~0	11	36.5	16.2	13.2	107.2
Oxygen	6.8	3.8	27.1	53	39.8	20.6	17.2	96.3
		8.9	64.4	124	38.0	17.1	14.9	96.3
		15.7	112.7	218	34.5	14.0	12.0	98.3
		26.5	190.7	401	30.4	12.0	13.0	94.0
Oxygen	5.7	357.0	28.0	43	29.7	9.7	9.7	107.0
		1190.0	93.2	140	23.5	4.2	7.4	94.8

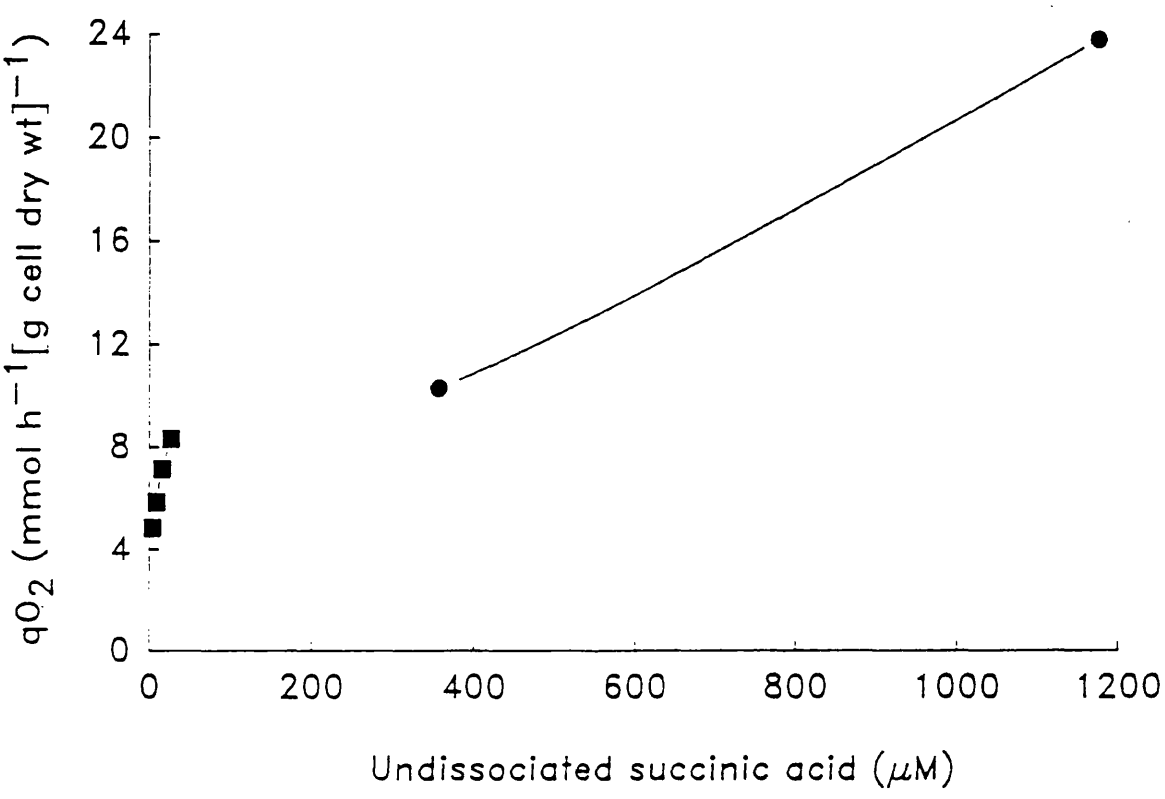


Figure 4.1. The effect of increasing undissociated succinic acid concentration on the respiration rate (qO_2) of an oxygen-limited continuous culture of *A. caulinodans*, (D = 0.10 h^{-1}, 30°C) at pH 6.8, (■) and pH 5.7, (●).

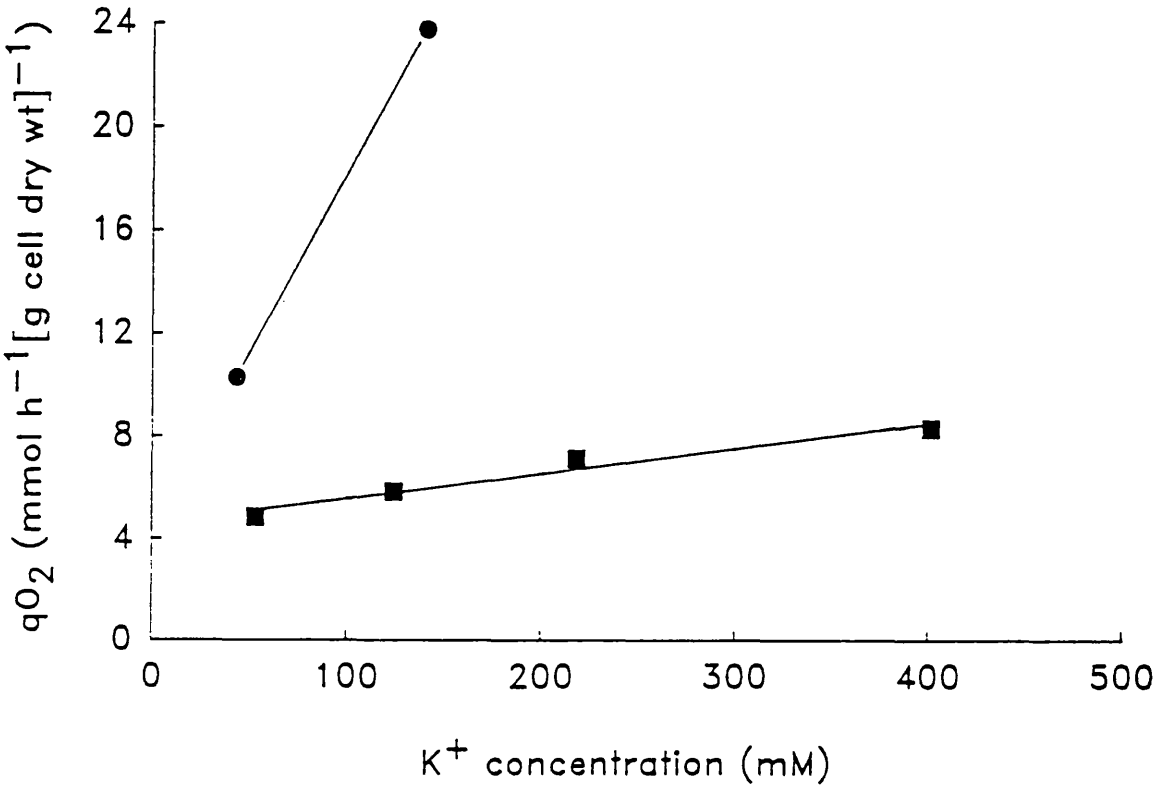


Figure 4.2. The effect of increasing K^+ concentration on the respiration rate (qO_2) of an oxygen-limited continuous culture of *A. caulinodans*, (D = 0.10 h^{-1}, 30°C) at pH 6.8, (■) and pH 5.7, (●).

Table 4.3. Molar growth yields for *A. caulinodans* grown under oxygen limitation in continuous culture at pH 6.8 and 5.7 (D = 0.03 h^{-1}, 30°C), were measured as described in Materials and Methods. The potassium ion concentration was maintained approximately constant by the addition of potassium chloride to the inflowing medium.

Culture pH	Steady-state concentration		Molar Growth Yields			
	$[H_2A]$ (μM)	K^+ (mM)	Ysucc (g cell dry wt mol^{-1})	Yo_2	Yco_2	Carbon Recovery (%)
6.8	7	225	30.9	16.7	11.5	98.1
	17	214	30.7	18.7	12.1	94.6
5.7	276	192	32.5	16.3	11.7	101.7
	1231	215	24.2	10.6	8.7	94.7

respectively, following a 4.5 fold increase in the concentration of undissociated succinic acid (276 to 1231 μM). The molar growth yields from cultures grown at pH 6.8 were unchanged by changes in undissociated succinic acid concentration from 7 to 17 μM. An undissociated succinic acid concentration of 276 μM in pH 5.7 culture yielded similar molar growth yields to cultures grown at pH 6.8 containing much lower undissociated succinic acid, suggesting that the undissociated succinic acid effects were not physiologically disruptive at this concentration. The observed growth yields were in very close agreement with the calculated values (derived from the stoichiometric growth equation).

pH *per se* appeared to have little effect ($\leq \pm$ 13%) on the molar growth yields of oxygen-limited cultures, in contrast to cells grown under succinate limitation where a decrease of up to 41% was observed. (Table 4.2). This was possibly due to the lack of capacity for respiration in succinate-limited cells to expel H^+.

Preliminary experiments aimed at measuring the rate of diffusion of undissociated [^{14}C]succinic and [^{14}C]acetic acid into washed cell preparations of *A. caulinodans* (harvested from oxygen-limited continuous cultures) were unsuccessful since high levels of non-specific substrate binding to the cells masked any evidence of diffusion.

4.3 Discussion

Hueting and Tempest (1977) found very low Yo_2 values for *Candida utilis* under acetate-excess growth conditions, and similarly for *A. caulinodans* the Yo_2 values varied markedly with the total extracellular concentration of the organic acid. These authors reported that when the total acetate concentration was raised progressively from 111 to 467 mM (pH 6.9), the Yo_2 decreased from 11.4 to 5.8 g cell dry wt mol^{-1}. When the extracellular acetate concentration was

further increased to 556 mM, growth was inhibited and the culture washed out.

The relationship between the Yo_2 and the extracellular concentration of unused acetate for *Candida utilis* (Hueting and Tempest, 1977), and succinate for *A. caulinodans* was also strongly influenced by the culture pH value. A fixed extracelluar acetate concentration of 356 mM ($D = 0.14\ h^{-1}$) and progressive lowering of the culture pH from 6.9 to 5.1 caused a decrease in the Yo_2 of *Candida utilis* (Hueting and Tempest, 1977). A further decrease of the culture pH to 4.8 resulted in a complete collapse of respiration, whereas, similar pH changes in acetate-limited culture only affected the Yo_2 slightly, and growth was possible at a pH value of 2.5.

The reason for the washout and cessation of growth at high extracellular succinate concentrations with *A. caulinodans*, was probably due to the inability of the cell to pump protons from the cytoplasm at a rate equal to that at which undissociated acid entered the cell. As a result the cytoplasm almost certainly became acidified and eventually death ensued; as with *Clostridium thermoaceticum* (Baronofsky *et al.*, 1984), and *Saccharomyces cerevisiae* (Pinto *et al.*, 1987) following growth on acetate.

In addition to the effects of undissociated succinic acid on the growth rate and molar growth yields of *A. caulinodans*, high concentrations of extracellular K^+ also exerted similar, but less pronounced effects. It is unclear how K^+ exerted the apparent growth retarding actions. However, interference of bacterial respiration due to precipitation of the proteins of the respiratory chain may have been a cause (Ingram, 1947). Salt-protein interactions may not be restricted to proteins involved in respiration but also to periplasmic and membrane bound proteins and so disrupt solute transport systems. Additionally, high extracellular salt levels may also interfere with the water potential of the cell causing osmotic stress.

The qO_2 of *K. aerogenes* was shown to increase as a function of

the extracellular K^+ concentration (Hueting *et al.*, 1979), and it was suggested that maintenance of a physiological potassium gradient was energetically expensive, and probably accounted for a large proportion of total energy expended in growth-unassociated functions. A similar conclusion may be applicable to *A. caulinodans*.

It is extremely unlikely that bacteroids would encounter extracellular K^+ concentrations as high as 400 mM, but the results presented here for *A. caulinodans*, show that care must be taken when interpreting growth yields from continuous cultures utilizing acidic substrates that require pH control with potassium hydroxide (and perhaps also with sodium hydroxide).

Unfortunately, very little data exist with respect to the pool size of organic acids of root nodules. Streeter (1987) determined the pool size for a wide range of metabolites including succinate, fumarate and malate which were present at 60, 92 and 539 μg g fresh weight nodule^{-1}. Assuming a cell volume of 0.73 μl mg^{-1} the calculated concentration of undissociated succinic acid, fumaric acid and malic acid at pH value 5.7 is 9.0 μM, 14.2 μM and 70.9 μM respectively. Clearly, if the metabolite concentrations reported by Streeter (1987) are true representations then the probability that organic acids act as uncoupling agents and have a deleterious effect on nitrogen fixation efficiency is low.

CHAPTER 5

Nitrogen-fixing growth of *Azorhizobium caulinodans* in continuous culture

5.1 Introduction

Despite numerous attempts (see Postgate, 1975) nitrogenase activity or growth on N_2 could not be detected in cultured free-living rhizobia for many years. These early failures led to the conclusion that an unidentified factor provided by the plant host was necessary for the expression of nitrogen-fixing genes in rhizobia. Dilworth and Parker (1969) suggested that initiation of the nitrogen-fixing process required the transfer of genetic information from the plant to the bacteroid. Although little if any genetic transfer occurs between the legume host and bacteroid, a considerable array of chemical messages are passed e.g. flavones and nodulins (Redmond *et al.*, 1986; Firmin *et al.*, 1986; see also Halverson and Stacey, 1986).

The first observations of nitrogen fixation in free-living (non-bacteroid) rhizobia came from experiments in which soybean cell cultures were inoculated with *B. japonicum* or cowpea-*Rhizobium* (Holsten *et al.*, 1971; Child and LaRue, 1974; Phillips, 1974), and nitrogen fixation was detected using the ethylene reduction test developed by Hardy *et al.* (1968). However, these initial reports were received with sceptism, since ethylene is a natural product commonly released from plant tissue. Unequivocal evidence of nitrogenase activity in free-living rhizobia, and so final disproof of host involvement, was not demonstrated until 1975, when several groups independently reported ethylene reduction by rhizobia cul-

tured in the absence of any plant material (Kurz and LaRue, 1975; Tjepkema and Evans, 1975; Keister, 1975; Pagan *et al.*, 1975).

Few reports have been published about the energy costs of nitrogen fixation in *ex-planta* rhizobia. Stam *et al.* (1984) reported an ATP/N_2 molar ratio (total amount of ATP use by nitrogenase during the fixation of 1 mol N_2) of 42 for *A. caulinodans* grown under succinate-limited conditions. The reported ATP/N_2 molar ratios of free-living diazotrophs under anaerobic conditions varies from 20 in carbon-limited *Clostridium pasteurianum* (Daesch and Mortensen, 1968) to 29 in carbon-limited *Klebsiella pneumoniae* (Hill, 1976).

Nitrogen fixation is always linked to proton reduction and consequently hydrogen evolution (Robson and Postgate, 1980). This hydrogen production represents a loss of energy and reducing equivalents as much as 30-60% of the electron flow through nitrogenase may be lost as hydrogen (Schubert and Evans, 1976). Many rhizobia, including *A. caulinodans* (Stam *et al.*, 1984), possess a membrane-bound uptake hydrogenase which enables them to reoxidise the formed hydrogen and to regain part of the energy lost (Carter *et al.*, 1978; Emerich *et al.*, 1979). Other possible functions of uptake hydrogenase are the removal of excess oxygen (Dixon, 1972) and the prevention of inhibition of nitrogenase by hydrogen (Dixon *et al.*, 1981).

Oxygen presents a paradox to aerobic diazotrophs in that it is required as the terminal electron acceptor for respiration (which conserves the energy required to sustain nitrogenase activity), but it prevents synthesis of nitrogenase and also inactivates existing nitrogenase proteins even at low oxygen concentrations. Bergersen (1977) observed nitrogenase activity with cowpea-*Rhizobium* at oxygen concentrations of 1 μM (0.4% DOT) but not at concentrations above 30 μM (12.5% DOT), and de Hollander (1981) reported nitrogenase activity at dissolved oxygen concentrations of 2-5 μM (0.8-2.1% DOT) for *R. trifolli*, but not at higher concentrations. Stam *et al.* (1983) reported nitrogenase activity for *R. leguminosarum* at very low oxygen concentrations (0-0.4% DOT). However, Stam *et al.* (1984) and

Gebhardt *et al.* (1984) reported nitrogen-fixing growth of *A. caulinodans* in continuous culture at DOT levels of between 12 and 17%, considerably higher than for any other rhizobia. Maintenance of continuous cultures of nitrogen fixing *A. caulinodans* required the construction of sophisticated DOT control systems.

The aim of the work presented in this chapter was to investigate the effect of oxygen on nitrogen-fixing *A. caulinodans*, and determine as far as possible the reasons for the reported high oxygen tolerance of this *Azorhizobium* spp.

5.2 Results

5.2.1 Development of a system for controlling the DOT of a culture by gas blending.

Investigation of the effect of oxygen on nitrogen-fixing growth of *A. caulinodans* in chemostat culture, required the design and construction of an accurate oxygen control system. This was carried out over a period of several months in collaboration with Mr S.F. Gallageher and Mr J.A. Dews (Shell Research Ltd, Sittingbourne, Kent), and a final system is described below.

The dissolved oxygen tension (DOT) in the chemostat was measured and controlled using an Ingold polarographic autoclavable oxygen probe (Ingold A.G, Industrie Nord, CH-8902, Urdorf, Switzerland), connected in series to a Chemap conditioning-amplifier (Alfa Laval A.G, Alteland Strasse 415, CH-8702 Mannendorf, Switzerland), (Fig 5.1).

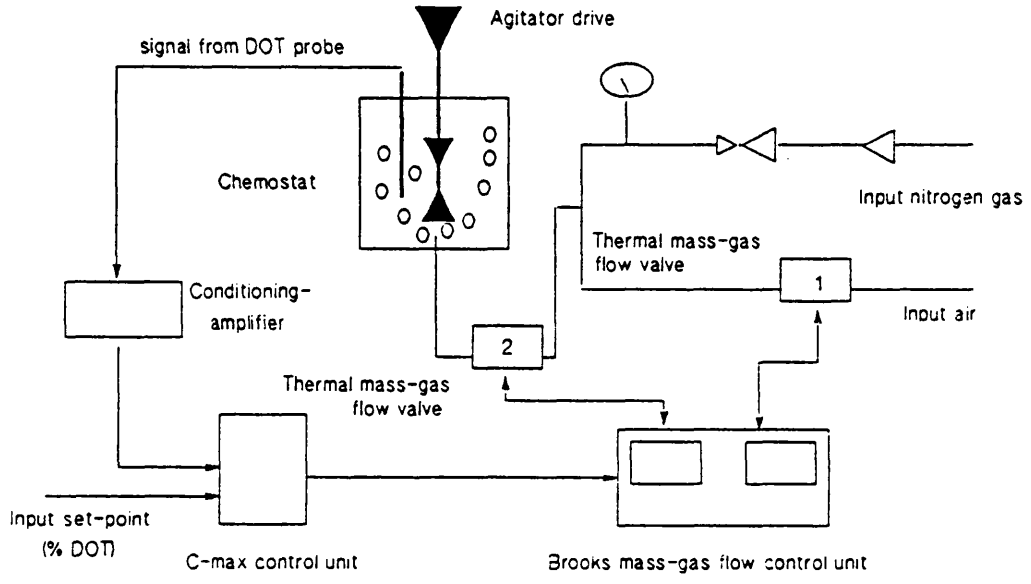


Figure 5.1. Schematic diagram of the dissolved oxygen control system used for regulating and maintaining a constant oxygen concentration in a chemostat.

The output from the conditioning-amplifier signalled the partial pressure of oxygen in the chemostat, and was relayed to a Leeds and Northrup "C-max" three-term control unit (Leeds and Northrup Ltd., Wharfdale Rd., Tynsley, Birmingham, U.K.). The C-max control unit calculated the margin of error from a pre-selected DOT set-point and initiated the appropriate corrective response. The C-max output regulated the opening of a Brooks Thermal Mass-gas valve (which use the thermal properties of the gas, as they are a function of gas mass, to calculate accurately the volume flow rates), *via* a Brooks Mass-gas controller (Brooks Instruments Ltd, Veenendaal, Holland), controlling the flow of air into a second Brooks Thermal Mass-gas flow valve. Maintenance of a constant mass-gas flow rate of 600 ml min^{-1} was achieved by mixing the inflowing air with nitrogen gas. The composition and flow rate of the gas entering the chemostat was controlled by a Hi-tec Mass Flow Control Unit (Krohne Measurement and Control Ltd. Northhampton, U.K.). The set-points of both the DOT and gas flow rate were selected manually.

Calibration of the dissolved oxygen control system was achieved by first determining the electrical zero point of the Ingold probe and then sparging the sterilized uninoculated culture medium (2.5l culture medium, pH 6.8, 30°C at 1.013 $x10^5$ Pa pressure and agitated at 600 rev. min^{-1}) with nitrogen gas; this state represented 0% DOT. The culture medium was then sparged with air under the same physical parameters as described for 0% DOT; this state represented 100% DOT (i.e. 100% of air saturation).

The % DOT value was converted into dissolved oxygen concentration by assuming that air-saturated medium at 30°C and 1.013 $x10^5$ Pa pressure contained 230 μM oxygen. The DOT value was stable to within 0.5% of the setpoint DOT to values of less than 1% DOT.

In preliminary experiments the dissolved oxygen control system was used to maintain the culture DOT at 44% during the ammonium assimilating batch growth of *A. caulinodans* (Fig 5.2). The growing culture had an oxygen demand approximately twice that which was

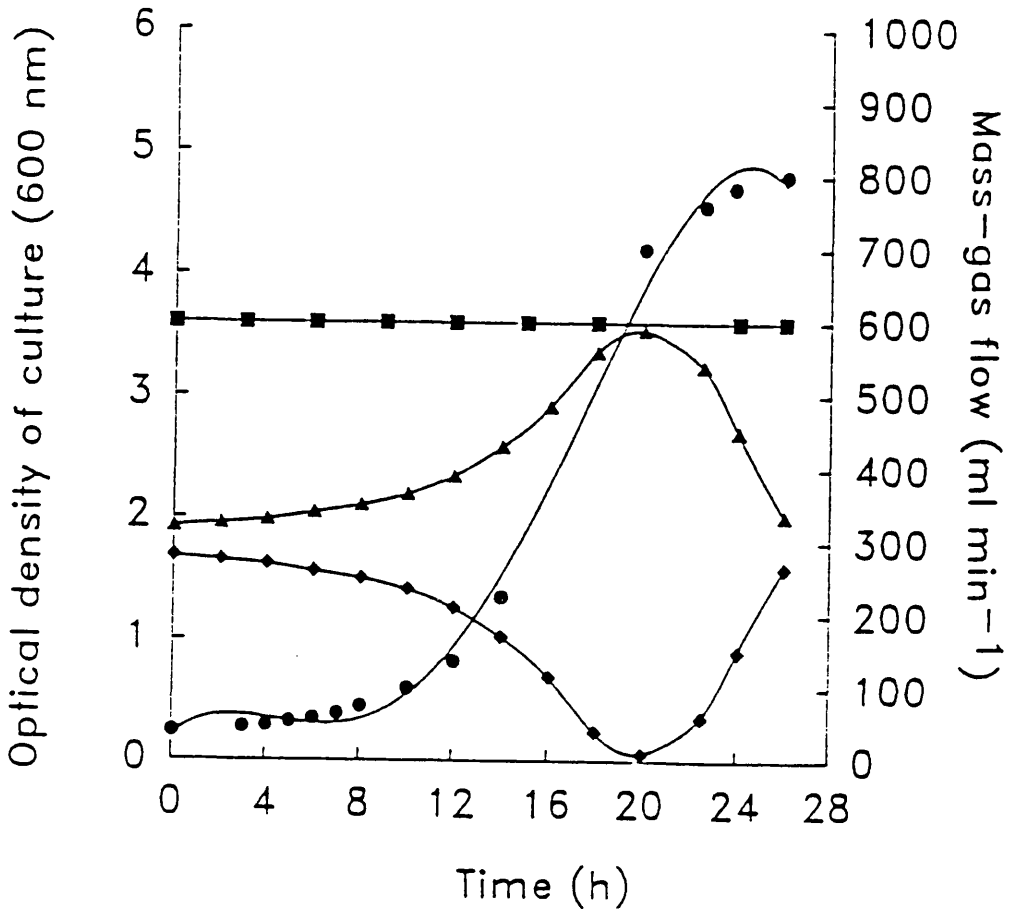


Figure 5.2. Ammonium-assimilating batch growth of *A. caulinodans* with succinate as the sole carbon source (pH 6.8, 30°C). The dissolved oxygen tension was maintained at 44% (± 0.5%) of air saturation, at a constant mass-gas flow of 600 ml min^{-1} by regulating the inflowing air : nitrogen gas ratio using the automatic gas blending system. Optical density of the culture at 600 nm, ● ;Air-flow, ▲; Nitrogen-flow, ◆ ; Total mass-gas flow, ■ .

supplied at the time of inoculation in order to maintain the set-point DOT (shown by the increasing air flow); however, the mass-gas flow remained constant at 600 ml min^{-1} as a result of the air : nitrogen ratio being altered under the control of the Hi-tec Mass Flow Control Unit. The DOT control system worked equally well at lower DOT setpoints between 1 and 5%.

Stam (1986) reported the regulation of *A. caulinodans* culture DOT at a constant value of 1 μM (0.4% DOT), by regulating the agitator rotation speed (using a continuous proportional and integrating controller with an integration time of 80 s). Similar systems had previously been used by Bergersen and Turner (1978, 1980), Ching *et al.* (1981) and Gebhardt *et al.* (1984). In all cases the gas flow remained constant but the culture volume almost certainly changed in response to the fluctuating agitator speed. However, marked changes in chemostat volume would seriously affect the dilution rate, and so make true steady-state conditions difficult or impossible to achieve.

Control of fermenter dissolved oxygen by the gas blending system described above allows steady-state to be achieved, eliminating the problems of volume changes by varying the gas composition.

5.2.2 Molar growth yields of *A. caulinodans* during growth under nitrogen-fixing sulphate-limited conditions and increasing DOT.

Molar growth yields for *A. caulinodans* grown under nitrogen-fixing conditions decreased as the oxygen concentration of the culture was increased over the range of 0% to 4% DOT (Table 5.1). At 4% DOT the specific growth rate of the culture declined to 0.07 h^{-1},

Table 5.1. Molar growth yields for *A. caulinodans* grown under nitrogen-fixing (oxygen/sulphate-limited) and ammonium assimilating (oxygen-limited) conditions in continuous culture (pH 6.8, 30°C), as described in Materials and Methods. The percentage dissolved oxygen of air saturation (DOT) was controlled by automatic gas blending.

Nitrogen source	Dilution rate (h^{-1})	Steady-state succinate (mM)	DOT (%)	Molar Growth Yields (g cell dry wt mol^{-1}) Ysucc	Yo_2	Yco_2	Carbon recovery (%)
N_2 (Oxygen-limited)							
	0.10	26.5	0	36.9	16.1	14.5	101.0
N_2 (Sulphate-limited)							
	0.10	25.5	1	29.9	12.1	9.5	105.4
	0.10	23.1	2	24.6	8.9	7.2	90.8
	(0.06[a]	17.1	4	25.9	10.5	8.8	98.6)
NH_4^+ (Oxygen-limited)							
	0.10	27.1	0	39.8	20.6	17.2	96.3

[a] = non steady-state.

and at 5% DOT it declined further to 0.025 h^{-1}; and in neither case was a satisfactory steady-state achieved. In contrast, Stam (1986) and Gebhardt *et al.* (1984) reported nitrogen-fixing growth of *A. caulinodans* at 17% and 5.2% DOT respectively, at a dilution rate of 0.10 h^{-1}. The reasons for this difference are unclear.

The measured $Y_{succinate}$ value of 36.9 g cell dry wt mol^{-1} (D= 0.10 h^{-1}) for nitrogen-fixing growth under oxygen limitation (0% DOT) was only slightly lower than the value of 39.8 g cell dry wt mol^{-1} following ammonium assimilating growth under the same conditions (Table 5.1), but confirmed the higher energy requirement for growth on nitrogen compared with combined nitrogen. Generally lower values were also reported by Stam (1986), *viz* 29.0 and 32.0 g cell dry wt mol^{-1} respectively. The molar growth yields on oxygen and with respect to carbon dioxide were also lower in nitrogen-fixing cultures compared to those assimilating ammonia (both oxygen-limited).

The $Y_{succinate}$ decreased from 36.9 to 24.6 g cell dry wt mol^{-1}, and the Yo_2 from 16.1 to 8.9 g cell dry wt mol^{-1} over the DOT range of 0 to 2% (D= 0.10 h^{-1}). Similar decreases in *A. caulinodans* growth efficiency in response to increasing oxygen concentrations were recorded by Stam (1986) and by Gebhardt *et al.* (1984), albeit over a much wider range of DOT. In the study by Stam (1986), the $Y_{succinate}$ decreased from 29.0 to 24.8 g cell dry wt mol^{-1} as the DOT increased from 0% to 17.0%. Unfortunately, no molar growth yields on oxygen or for carbon dioxide were also reported. Decreased Yo_2 values (25.8 to 7.6 g cell dry wt mol^{-1}) were reported by Gebhardt *et al.* (1984) following growth on lactate at 1.7 and 5.2% DOT. Unfortunately, no other growth yield measurements were noted.

It is unclear how Stam (1986) maintained nitrogen-fixing growth at D= 0.10h^{-1} with a DOT value of 17%, as Gebhardt *et al.* (1984) reported that 12.8% DOT was close to the maximum oxygen tolerance limit. The results presented above suggested that *A. caulinodans* was unable to tolerate a DOT above 2% and still maintain a specific

growth rate of $0.10h^{-1}$. The high calculated Yo_2 value of 9.3 g cell dry wt mol^{-1} (qO_2 = 10.7 mmol oxygen h^{-1}) at 17% DOT derived from Stam (1986), and an observed Yo_2 value of 7.6 g cell dry wt mol^{-1} (qO_2 = 13.2 mmol oxygen h^{-1}) at 5.2% DOT from Gebhardt *et al.* (1984) were similar to the qO_2 of 11.2 mmol oxygen h^{-1} reported here for *A. caulinodans* following growth at 2% DOT (D=0.10 h^{-1}). The medium composition in all three cases were very similar, and it is unlikely that the observed differences were a result of small differences in the availability of iron, molybdenum or nicotinic acid concentrations. Neither were there significant differences in the culture pH or temperature.

Elemental analysis of *A. caulinodans* following nitrogen-fixing growth under oxygen limitation (D = 0.10 h^{-1}) showed a cellular nitrogen content of 9.5%, and yielded a cell formula of $C_1H_{1.76}N_{0.17}O_{0.55}P_{0.01}$. The nitrogen content of cells grown under the same conditions but with ammonia as the nitrogen source was higher at 11.5%, and yielded a cellular formula of $C_1H_{1.67}N_{0.21}O_{0.51}P_{0.01}$. Elemental analysis of nitrogen-fixing *A. caulinodans* cells reported by Stam (1986) showed a nitrogen content of 10.1%, i.e. similar to the value reported above.

The specific rate of nitrogen fixation (qN_2) calculated from the nitrogen content of the cells during oxygen-limited growth (D = 0.10 h^{-1}) was 489 μmol N_2 h^{-1} (g cell dry wt)$^{-1}$ (corrected for the nitrogen content of the growth medium), and was in close agreement with the value of 513 μmol N_2 h^{-1} (g cell dry wt)$^{-1}$ calculated from the data of Stam (1986). These values were slightly higher than the qN_2 values from Gebhardt *et al.* (1984) which ranged from 213 to 378 μmol N_2 h^{-1} (g cell dry wt)$^{-1}$ (at 0.6% and 2.5% DOT respectively). It is unclear why the qN_2 values of Gebhardt *et al.* (1984) increased with increasing oxygen tension.

From the comparison of ammmonium-assimilating oxygen-limited cultures with nitrogen-fixing oxygen-limited cultures, the apparent ATP/N_2 molar ratio can be calculated from the Y_{ATP} values and the

amounts of N_2 fixed according to the equation of Hill (1976);

Apparent ATP/N_2 molar ratio

$$= \frac{Y_{ATP}(NH_4^+) - Y_{ATP}(N_2)}{Y_{ATP}(NH_4^+)} \times \frac{28}{F \times Y_{ATP}(N_2)}$$

in which the $Y_{ATP}(NH_4^+)$ and $Y_{ATP}(N_2)$ are the Y_{ATP} values for the ammonium and nitrogen fixing cultures ($D=0.10h^{-1}$) respectively, and F represents the percentage of nitrogen per g dry wt in the nitrogen-fixing culture. The $Y_{ATP}(NH_4^+)$ values calculated using the equation of Pirt (1975) (see section 3.2.6) using a Yo_2 value of 20.6 g cell dry wt mol^{-1} (see Table 3.7), were 10.3, 5.15 and 3.43 g cell dry wt mol^{-1} for P/O quotients of 1, 2 and 3 respectively. The calculated $Y_{ATP}(N_2)$ values using a Yo_2 value of 16.1 g cell dry wt mol^{-1}, were 8.05, 4.03 and 2.68 g cell dry wt mol^{-1} for P/O quotients of 1, 2 and 3 respectively. The percentage of nitrogen per g (F) in the nitrogen-fixing culture was 9.5%. Substitution of these values in to the above equation yielded apparent ATP/N_2 molar ratios of 8.0, 16.9 and 24.0 for P/O quotients of 1, 2 and 3 respectively. Similar values for the ATP/N_2 ratio were obtained using the equation of Stam (1986).

Comparison of an ammonia-assimilating succinate-limited culture of *A. caulinodans* (Yo_2 = 27.0, Y_{ATP} = 6.0 g cell dry wt mol^{-1}) with a nitrogen-fixing succinate-limited culture (Yo_2 = 12.1, Y_{ATP} = 3.13 g cell dry wt mol^{-1}) with an H_2/N_2 ratio of 7.46 (both growing at 0.10 h^{-1}) by Stam (1986) resulted in an apparent ATP/N_2 molar ratio of 42, markedly higher than the value reported here for oxygen-limited *A. caulinodans*.

5.2.3 Cytochrome content of *A. caulinodans* during growth under nitrogen-fixing conditions and increasing DOT.

Analysis of the cytochrome spectra of cells harvested from nitrogen-fixing cultures grown at 0 (oxygen-limited; $D=0.10h^{-1}$) and 5% DOT (sulphate-limited; $D=0.025h^{-1}$), showed a marked change in the ratio of total *c*-type to total *b*-type cytochrome (Fig 5.3). The cytochrome *c* concentration decreased from 243 pmol mg cell dry wt^{-1} at 0% DOT to 136 pmol mg cell dry wt^{-1} at 5% DOT, whereas the cytochrome *b* concentration decreased from 180 pmol mg cell dry wt^{-1} at 0% to 109 pmol mg cell dry wt^{-1}. As a result the total *c* to total *b* ratio declined from 1.35 to 1.25 : 1. The concentration of cytochrome oxidase aa_3 did not appear to change between 0 and 5% DOT.

The only occasion where cytochrome *d* (absorbance maxima 630 nm) was observed in *A. caulinodans* was from a 48 h batch culture growing initially on ammonia as the sole nitrogen source, however, following a 24 hour failure of the agitator after approximately 15-20 h growth, the cell density was found to be approximately two fold higher than the medium nitrogen could support. This observation strongly suggested that the culture was fixing nitrogen. Cytochrome *c* was virtually undetectable in these cells, in contrast to cytochrome *b* (Fig 5.3).

5.3 Discussion

The successful development of a system for accurately controlling the DOT of a nitrogen-fixing culture at very low levels by gas blending allowed accurate measurement of the molar growth yields on

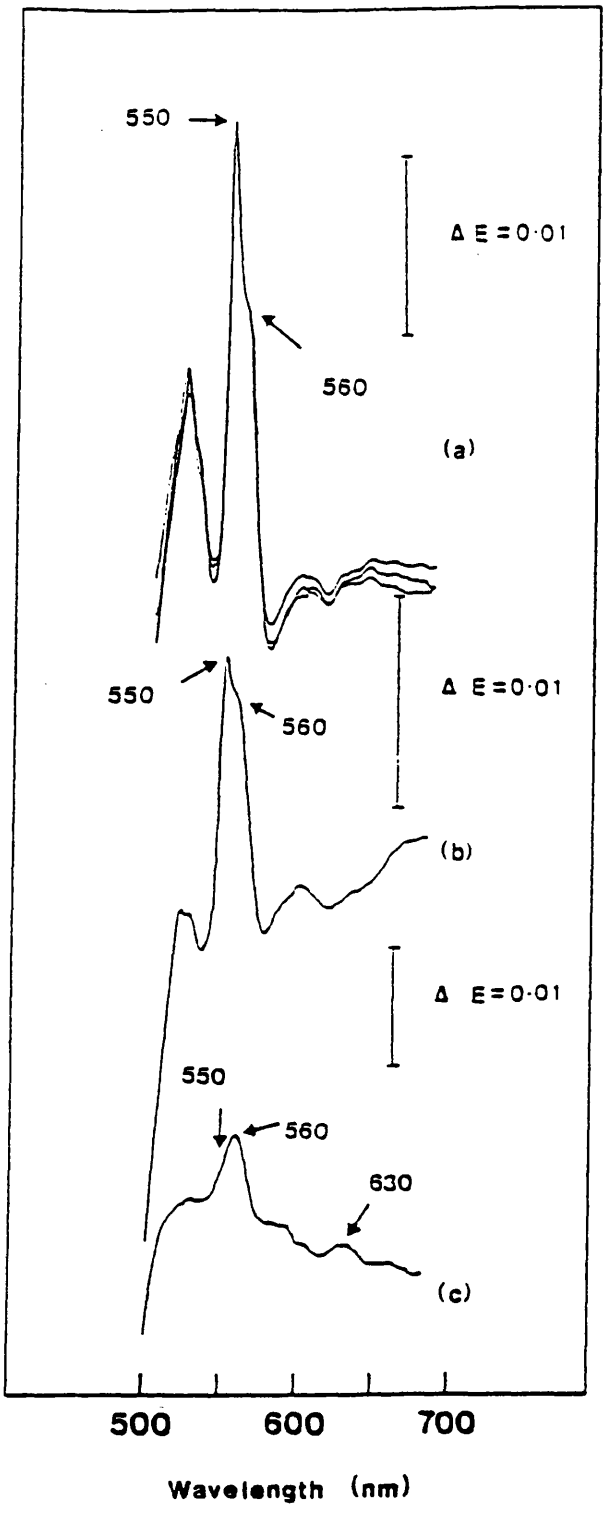


Figure 5.3. Dithionite-reduced *minus* ferricyanide oxidised difference spectra of *A. caulinodans* grown in continuous culture at various dissolved oxygen tensions, under N_2-fixing conditions, with oxygen and sulphate as the growth limiting nutrient ($D=0.10h^{-1}$ and $0.025h^{-1}$ respectively). Broken cells (6 mg cell dry wt) were prepared as described in Materials and Methods. % DOT (a) 0%, (b), 5%, (c) "N_2-fixing" cells from an initially NH_4^+ grown culture $\pm$10% DOT.

oxygen and carbon dioxide by maintaining a constant mass-gas flow through the chemostat. The system also prevented fluctuations in the chemostat volume as the agitator speed remained constant, and so maintained a constant dilution rate during steady-state measurements. The low degree of "system-error" (± < 0.5% DOT) during operation ensured accurate DOT control around the set-point.

The molar growth yields for *A. caulinodans* grown under oxygen limitation were lower following nitrogen-fixing growth than following ammonia-assimilating growth under the same conditions, confirming the higher energy requirement for growth on nitrogen gas compared to combined nitrogen. Little data is available about the molar growth yields of nitrogen-fixing cultures of *Rhizobium*, possibly due to the difficulty in derepressing nitrogenase activity. However, similar results were obtained for *R.leguminosarum* where a large decrease in the $Y_{mannitol}$ from 33 to 15 g cell dry wt mol^{-1} was observed following a shift from ammonium-assimilating to nitrogen-fixing growth at 0.4% DOT (Stam *et al.*, 1983), and Stam *et al.* (1984) reported significant differences in the $Y_{succinate}$ and Yo_2 between succinate-limited ammonium-assimilating and nitrogen-fixing *A. caulinodans* grown at 17% DOT under the same conditions.

The nitrogen-fixing molar growth yields also decreased as the DOT of the culture was increased, the $Y_{succinate}$ and Yo_2 decreasing by 18 and 25% respectively as the DOT increased from 0 to 1% and similarly from 1 to 2% ($D = 0.10h^{-1}$). These results suggested that increasing oxygen concentration was decreasing the growth efficiency of nitrogen-fixing cultures. Similar changes in molar growth yields were reported by Stam (1986) and Gebhardt *et al.* (1984) but not discussed.

Few reports have been published on the energy costs of nitrogen-fixation in *ex planta* rhizobia. The reported ATP/N_2 molar ratio in free-living anaerobic diazotrophs varies from 20 for *Clostridium pasteurianum* (Daesch and Mortenson, 1968) to 30 for *Klebsiella pneumoniae* (Hill, 1976; see Phillips, 1980). The ATP/N_2 molar ratio

of 17-24 reported here for oxygen-limited *A. caulinodans* (assuming a P/O quotient of 2 and 3 respectively) was similar to the above values but lower than the value of 42 for succinate-limited *A. caulinodans* reported by Stam (1986) (assuming a P/O quotient of 2 and an H_2/N_2 ratio of 7.5).

Nitrogen-fixing growth under oxygen-limited conditions may represent the state of maximum efficiency for nitrogenase in aerobic diazotrophs in terms of the ATP/N_2 molar ratio, as the calculated values for *A. caulinodans* of 17 (assuming a P/O quotient of 2) was similar to the values of between 12 and 16 calculated from the reported values of ATP requirement for electron transfer by nitrogenase preparations from various organisms (Zumft and Mortensen, 1975; Haaker and Klugist, 1987). However, such comparisons maybe misleading because the amount of ATP hydrolysed per electron pair transferred can be influenced by the ratio of the two nitrogenase components and the assay conditions (Zumft and Mortenson, 1975; see Hill, 1976).

Increasing the culture DOT of *A. caulinodans* from 0% to 1% resulted in a decrease in the calculated Y_{ATP} from 4.03 to 3.03 g cell dry wt mol^{-1} and at 2% the calculated Y_{ATP} was 2.25 g cell dry wt mol^{-1} (assuming a P/O quotient of 2). Comparing these values to those from an aerobic ammonium-assimilating culture (Yo_2 = 27, Y_{ATP} = 6 g cell dry wt mol^{-1}) the apparent ATP/N_2 molar ratio increases from 17 (oxygen-limited) to 48 at 1% DOT and 82 at 2% DOT. The high ATP/N_2 molar ratio of 42 reported by Stam *et al.* (1984) compared to the value of 17 reported here for oxygen-limited *A. caulinodans*, may have been a due to the effects of oxygen. The low Yo_2 (12.1 g cell dry wt mol^{-1}; qO_2 = 8.3 mmol h^{-1} [g cell dry wt]$^{-1}$) from Stam *et al.* (1984) clearly showed an increase in respiration rate compared to ammonium-assimilating cultures. These increased respiration rates may have served to prevent nitrogenase inactivation by oxygen and/or nitrogenase reduction of oxygen to form superoxide radicals ($O_2^{\cdot -}$) which could have serious consequences on cell integrity (Puppo *et*

al., 1986).

However, the apparent ATP/N_2 molar ratio is totally dependent upon the calculated Y_{ATP} value. Increasing the difference between the Y_{ATP} (NH_4^+) and Y_{ATP} (N_2) increases the ATP/N_2 value, and the validity of the estimate of Y_{ATP} in nitrogen-fixing cultures depends upon the assumption that yield of ATP from succinate dissimilation is the same as during growth on combined nitrogen.

Calculations of ATP/N_2 molar ratios for aerobic diazotrophs are further complicated by hydrogen production and whether or not hydrogen re-oxidation occurs. Hydrogen production has a marked effect on ATP/N_2 molar ratios since it obviously consumes ATP. Hydrogen reoxidation yields less ATP than NADH oxidation (Stam *et al.*, 1984) and calculation of the Y_{ATP} becomes increasingly difficult and dependent upon accurate knowledge of the *in vivo* hydrogenase activity. Expression of hydrogenase under oxygen-limited conditions is energetically unfavorable (see Stam *et al.*, 1984) and so the calculated Y_{ATP} values used to derive the ATP/N_2 molar ratio for *A. caulinodans* reported here are not complicated by hydrogenase activity.

Analysis of the cytochrome spectra of cells of *A. caulinodans* harvested from nitrogen-fixing cultures grown between 0 and 5% DOT, showed the presence of *c*- and *b*- type cytochromes. The nature of the terminal oxidase was unclear, although spectral analysis suggested the presence of terminal oxidase aa_3 between 0% and 5% DOT. However, under ammonium-assimilating oxygen-limited growth conditions cytochrome oxidase aa_3 was not detected (see chapter 3), and cytochrome oxidase *d* was only positively detected in non steady-state "nitrogen-fixing" cultures grown at an approximate DOT of 10%, although the oxygen tension at the cell surface may have been considerably lower due to the lack of agitation. The disappearance of the aa_3 peak following nitrogen-fixing growth and synthesis of cytochrome *d*, has been previously reported (Stam *et al.* 1984) although no evidence was presented, and the DOT of the nitrogen-fixing culture not recorded.

The reasons for the high tolerance to oxygen during nitrogen-fixing growth of *A. caulinodans* compared to that of other rhizobia (Bergersen, 1976; de Hollander, 1981; Stam *et al.*, 1983) are unclear. Both stem and root nodules of *Sesbania rostrata* containing *A. caulinodans* have been found to contain substantial amounts of leghaemoglobin. However, the potential benefits of leghaemoglobin are unavailable to free-living cells. The increased respiration rate (decreased Yo_2) of nitrogen-fixing cells observed following increases in culture DOT was similar to the response already documented for *Azotobacter* spp. (Drozd and Postgate, 1970; Jones *et al.*, 1973). It is possible that some form of respiratory protection of nitrogenase similar perhaps to that of *Azotobacter* may exist in nitrogen-fixing *A. caulinodans*, however, the cytochrome content of cells cultured between 1 and 5% DOT did not obviously contain cytochrome *d*. The latter was only observed in a non steady-state "nitrogen-fixing" culture, which expressed a cytochrome pattern very similar to that found in *Azotobacter* spp. showing respiratory protection (Jones, 1973). However, care must be taken when interpreting these results due to the nature of the experimental conditions.

Despite the presence of leghaemoglobin and the low reported oxygen concentrations found in soybean nodules ($\leq$ 10 nm) (Bergersen, 1982; Appelby, 1984), uncoupling of respiration and energy production has been reported in bacteroids of *B. japonicum* (Bergersen and Turner, 1975; Appelby, 1984) and interpreted as respiratory protection of nitrogenase. Alternative strategies to protect nitrogenase; conformational change and compartmentalisation (*cf.* heterocysts in cyanobacteria) are unlikely to occur since the former would inactivate nitrogenase and the latter is not commonly found in bacterial systems.

High levels of superoxide dismutase were reported in *A. caulinodans* bacteroids isolated from root and stem nodules of *Sesbania rostrata*, and similar enzyme patterns were also seen in the free-living bacterial cells (Puppo *et al.*, 1986). However, the probable

role of superoxide dismutase would be to remove oxygen free-radicals ($O_2^{\cdot -}$), possibly generated from the reduction of oxygen by nitrogenase, or protect leghaemoglobin against accidental autoxidation (Puppo *et al.*, 1982).

The reasons for the higher degree of oxygen tolerance of *A. caulinodans* compared to other rhizobia is still unclear and further work is required to characterise this phenomenon.

CHAPTER 6

C_4-dicarboxylate incorporation and metabolism in *A. caulinodans*

6.1 Introduction

Bacteria often inhabit environments where nutrients are in short supply, and different species must compete with each other for the available carbon sources. Free-living rhizobia can grow on at least 20 different carbon substrates ranging from sugars to organic acids (Stowers, 1985), which enter the cell either in a passive manner through simple or facilitated diffusion, or alternatively the uptake is "active", requiring the expenditure of metabolic energy to transport carbon substrates and achieve intracellular concentrations sufficient for optimal growth. Strategies requiring the expenditure of energy for the initial transport of substrates across the cytoplasmic membrane fall into two general classes; firstly phosphorylation systems for example using phosphoenolpyruvate or ATP, and secondly, gradient systems where transport may be energised either by a gradient of H^+, or Na^+ (K^+, Ca^{2+} *etc.*). The majority of nutrients, especially when the external concentrations are low, enter the cell *via* an active process.

The carbon nutrition of bacteroids is determined by the plant host. Sucrose is the major carbon product exported from photosynthesising plant chloroplasts, following the breakdown of starch and also the principal carbon product to be transported into leguminous root nodules, but is not a major carbon substrate utilised by symbiotic nitrogen-fixing bacteriods (Stowers, 1985). Mutant strains of

R. leguminosarum (Glenn *et al.*, 1984; Mckay *et al.*, 1985), *R. meliloti* (Duncan, 1981), and *R. leguminosarum* (biovar *trifolii*) (Ronson and Primrose, 1979), that were defective in sugar metabolism were all capable of fixing atmospheric nitrogen. Bacteroids of *R. leguminosarum* unable to incorporate glucose (Hudman and Glenn, 1980; de Vries *et al.*, 1982), or oxidise glucose (Glenn and Dilworth, 1981), were also found to be capable of nitrogen fixation. Furthermore, Saroso *et al.* (1984), reported the presence of a relatively sugar-impermeable peri-bacteroid membrane in a strain of cowpea-*Rhizobium*.

The ability of rhizobia to catabolise sugars is therefore not necessarily required for nitrogen fixation *inplanta*, in contrast to a functional tricarboxylic acid cycle and C_4-dicarboxylic acid transport system (Bergersen and Turner, 1967). Mutant strains of *R. leguminosarum* (Finan *et al.*, 1981), and *R. meliloti* (Gardiol *et al.*, 1982) deficient in succinate and malate dehydrogenase activity or alternatively 2-oxoglutarate dehydrogenase activity (Duncan and Fraenkel, 1979) formed ineffective nodules. Mutant strains of *Rhizobium* unable to transport C_4-dicarboxylic acids (*Dct*$^-$), in the bacteroid form; *R. leguminosarum*, (Glenn *et al.*, 1980; Finan *et al.*, 1981 and 1983; Arwas *et al.*, 1985), and *Bradyrhizobium japonicum* (San Francisco and Jacobsen, 1985), were also Fix$^-$. Ronson *et al.* (1981), reported that free-living strains of *R. trifolii* defective in C_4-dicarboxylate transport were unable to enter into an effective symbiosis with Alfalfa.

Studies of dicarboxylic acid transport in *R. leguminosarum* (Finan *et al.*, 1981), *B. japonicum* (McAllister and Lepo, 1983; San Francisco and Jacobsen, 1985), and *R. meliloti* (Bolton *et al.*, 1986; Engelke *et al.*, 1987), have shown that the three major C_4-dicarboxylates found in plants (fumarate, malate and succinate), are all transported by a common C_4-dicarboxylate transport system. The transport system is believed to be a proton-coupled mechanism (symport) in which respiration or ATP hydrolysis first generates a proton gradient, and proton re-equilibration is then coupled to

dicarboxylate uptake. Indirect evidence for involvement of a proton gradient was founded on the observation that the addition of artificial protonophores (uncoupling agents) markedly inhibit dicarboxylate incorporation in a number of rhizobia (Glenn *et al.*, 1980; San Francisco and Jacobsen, 1985). The system is also believed to be inducible in many rhizobia (Ronson *et al.*, 1981; Finan *et al.*, 1981; McAllister and Lepo, 1983), although a constitutive system has been reported for two *Bradyrhizobium* species (Glenn *et al.*, 1980).

Considerable recent progress has been made towards understanding the organisation and regulation of the genes involved in C_4-dicarboxylic acid transport (*dct*). Initial studies by Ronson *et al.*, (1984), suggested the existence of two *dct* loci (designated A and B) located within a a 5.5 kilobase region of chromosmal DNA in *R. leguminosarum*. The two initial loci have now been resolved into three genes, *dct* A, from locus A and *dct* B and D both from locus B. The gene product of *dct* A (Dct A), is the structural component of the C_4-dicarboxylate transport system, and believed to be a hydrophilic polypeptide containing 444 amino acid residues, 68% of which are non-polar suggesting strong membrane associations. The transport system is believed to be regulated by both Dct B and Dct D, where Dct B is a protein kinase. The protein kinase is activated when bound to a C_4-dicarboxylic acid and phosphorylates Dct D. Activated Dct D in conjunction with the sigma factor rpoN and an RNA polymerase initiates the transcription of Dct A (Ronson, 1988). The precise mechanism of *dct A* activation is still unknown, with emerging evidence suggesting that factors controlling activation are different in free-living cells and bacteroids (Ronson, 1988).

Unfortunately, the nomenclature and description of experimental results for many radio-labelled C_4-dicarboxylate incorporation studies in the literature are often misleading. The term "transport-rate" of the radio-labelled substrate is frequently used to describe the rate of metabolism, and "K_m" values are often presented that are derived from such rates. In this thesis, the term "transport" is

reserved for the measurement of the initial entry of the substrate into the cell before the rate becomes determined by subsequent metabolism.

This chapter describes the investigation and characterisation of the various factors regulating C_4-dicarboxylate metabolism in free-living cells of *A. caulinodans* ORS 571.

6.2 Results

6.2.1 [^{14}C]succinate incorporation and disappearance.

The rate of succinate incorporation by whole cells of *A. caulinodans* grown in succinate-limited continuous culture was measured using 2,3-[^{14}C]succinate. The method used (see Materials and Methods) was similar in many respects to those described earlier for other rhizobia (Glenn *et al.*, 1980; Hudman and Glenn, 1980; Finan *et al.*, 1981; San Francisco and Jacobsen, 1985; Bolton *et al.*, 1986; Engelke *et al.*, 1987; Birkenhead *et al.*, 1988), all of which involved entrapment of the cells on membrane filters post-incubation with a radio-labelled substrate. The amount of radio-label present in the trapped cells was then quantified using scintillation counting.

In initial experiments, the nitrocellulose filters used for

cell entrapment, were washed twice with buffer, then dried under a heating lamp (Finan *et al.*, 1981, San Francisco and Jacobsen, 1985 and Birkenhead *et al.*, 1988), for 20 min prior to immersion in scintillation fluid. The measured rate of [^{14}C]succinate incorporation using this "dry assay" procedure (Fig 6.1) was much lower than the $q_{succinate}$ value *in vivo* (40 nmol min^{-1} [mg cell dry wt]$^{-1}$), presumably due to extensive metabolism of the succinate to carbon dioxide during the drying phase. This procedure was therefore subsequently modified such that the filters were immersed in the scintillation fluid immediately after washing (Bolton *et al.*, 1986). The measured rates of [^{14}C]succinate incorporation using this "wet assay" procedure were at least equal to the *in vivo* $q_{succinate}$ value (67 ct 40 nmol min^{-1} [mg cell dry wt]$^{-1}$), and were therefore physiologically significant. The "wet assay" was therefore used in all subsequent experiments unless stated otherwise. The measured initial rate of [^{14}C]succinate incorporation was linear over a period of 80s and was fully saturated at 250 μM succinate for all nutrient limitations studied.

The rate of [^{14}C]succinate incorporation by washed cells was not increased when either glucose (5mM) or ethanol (1mM) were added prior to [^{14}C] succinate, thus indicating that initial oxidation of the transported succinate or that the endogenous reserves of the washed cells were sufficient to energize transport fully. The [^{14}C]succinate incorporation rate remained linear for up to 30 min (Fig 6.2). As similar rates were obtained when assayed over 30 min or 80s these results indicated that even the 80s rates probably reflected the further metabolism of succinate rather than its rate of transport *per se*.

The rate of [^{14}C]succinate disappearance from the reaction medium of cells grown under succinate-limited conditions at D=0.065 h^{-1} (Fig 6.3) also remained constant over a 30 min period and was approximately equal to the rate of [^{14}C]succinate incorporation (Fig 6.4). The disappearance of [^{14}]succinate from the reaction mix like

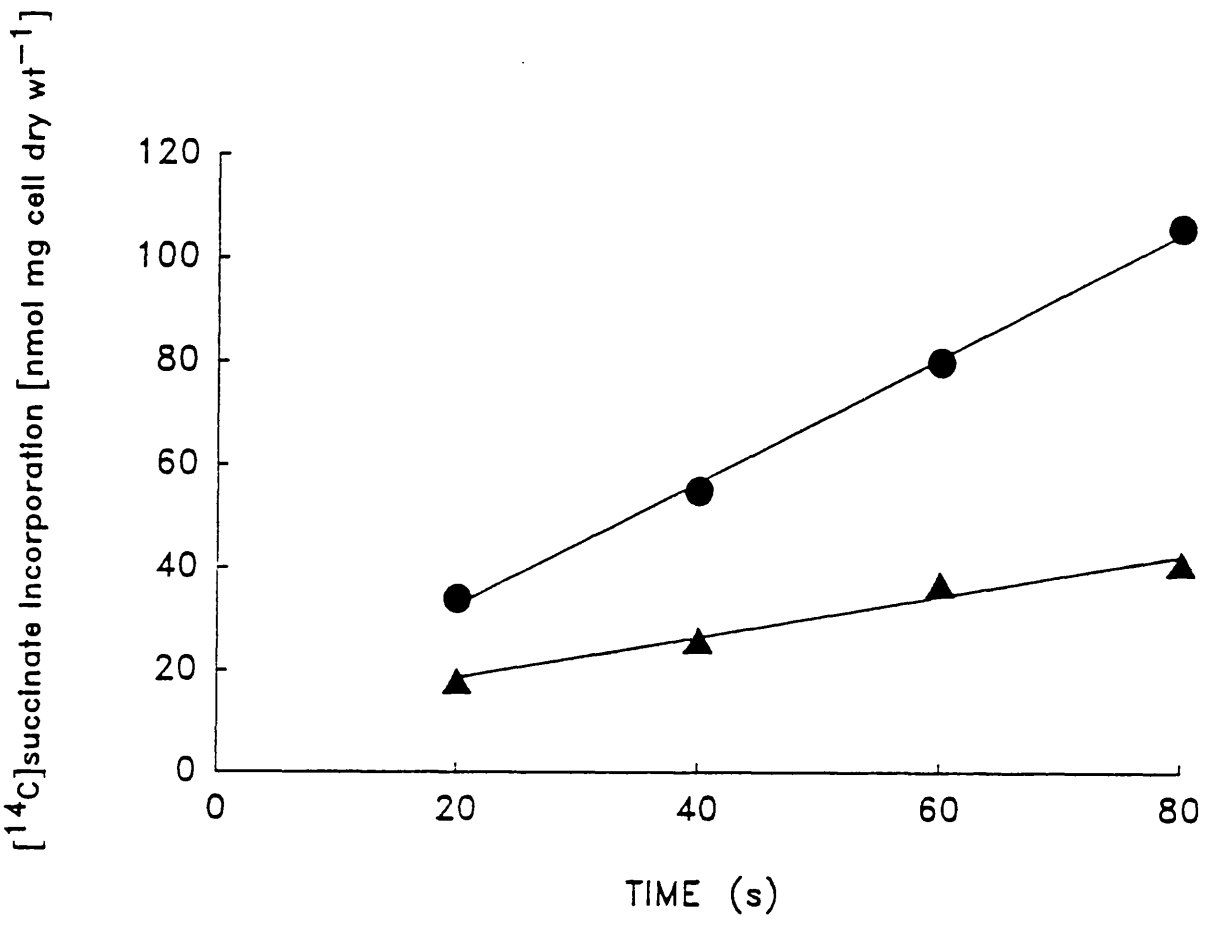


Figure 6.1. Incorporation of [^{14}C]succinate into washed cells of *A. caulinodans* grown under succinate limitation at D=0.1h^{-1}. [^{14}C]succinate incorporation was measured using either the dry assay procedure, ▲ or the wet assay procedure ● , as described in Materials and Methods. The rates were 27.1 and 66.7 nmol min^{-1} (mg cell dry wt)$^{-1}$ respectively. The *in vivo* $q_{succinate}$ was 40 nmol min^{-1} (mg cell dry wt)$^{-1}$.

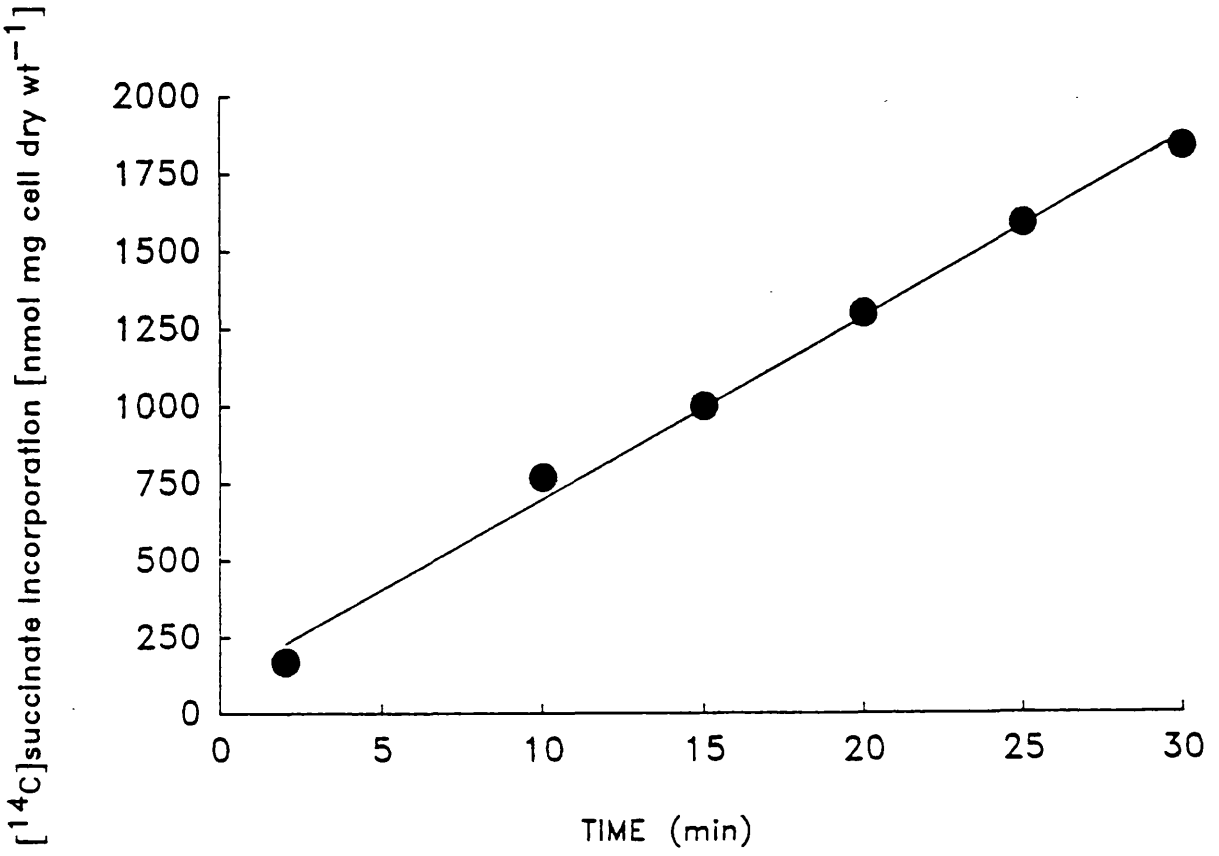


Figure 6.2. Incorporation of [^{14}C]succinate into washed cells of *A. caulinodans* grown under succinate limitation at D=0.065h^{-1}. [^{14}C]succinate incorporation was measured using the wet assay procedue as described in Materials and Methods, and occurred at a rate of 57 nmol min^{-1} (mg cell dry wt)$^{-1}$.

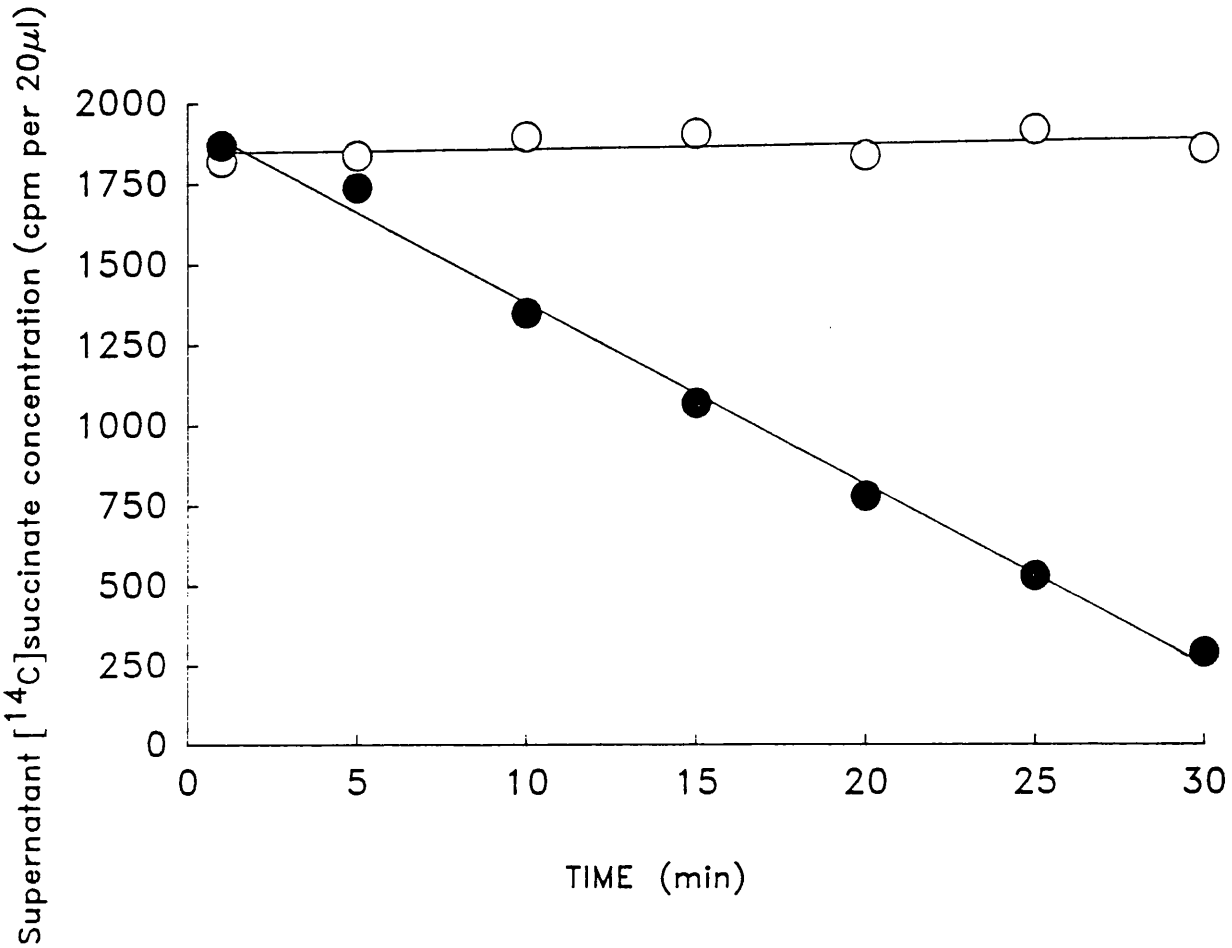


Figure 6.3. Disappearance of [^{14}C]succinate from supernatants of washed cells of *A. caulinodans* grown under succinate limitation at D=0.065h^{-1}. Cell suspensions were pre-incubated with or without FCCP (20 μM) for 1 min prior to [^{14}C]succinate addition. [^{14}C]succinate disappearance was measured as described in Materials and Methods, and occurred at a rate equivalent to an incorporation rate of 59 nmol min^{-1} (mg cell dry wt)$^{-1}$. Succinate, ● ; Succinate + FCCP, ○ . (cpm : counts per minute).

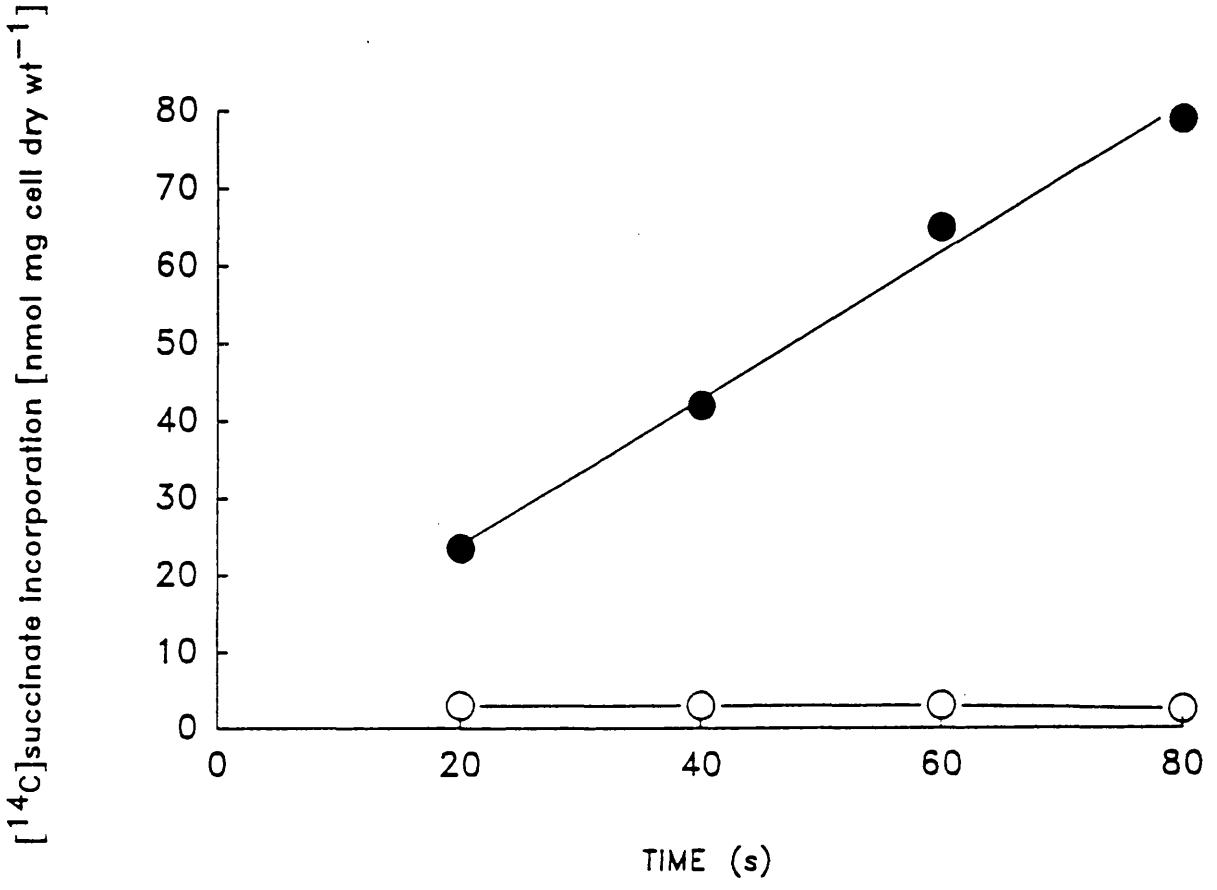


Figure 6.4. Incorporation of [^{14}C]succinate into washed cells of *A. caulinodans* grown under succinate limitation at D=0.065 h^{-1}. [^{14}C]succinate incorporation was measured using the wet assay procedure as described in Materials and Methods. the rate was 56.7 nmol min^{-1} (mg cell dry wt)$^{-1}$. Succinate, ● ; Succinate + FCCP (20μM), ○ .

its incorporation was completely inhibited by the uncoupling agent FCCP (20 μM).

Measurement of the rate of [^{14}C]succinate incorporation as a function of succinate concentration over the range 20 to 600 μM indicated that the transport and/or subsequent metabolism of [^{14}C]succinate, was saturated at 20 μM succinate (30°C). Lower succinate concentrations were tested, but the rate of incorporation was too fast for reproducible rates to be measured. Efforts to resolve this problem by increasing the concentration of cells and increasing the specific activity of the [^{14}C]succinate yielded highly variable results and so were unsuccessful.

The results obtained above with *A. caulinodans* clearly showed that the concentration of succinate (250 μM) routinely used in these assays was sufficient to saturate the system.

The "Km" value for succinate incorporation by *A. caulinodans* (<20 μM), was in accordance with "Km" values previously reported for *Ps. putida* and various species of *Rhizobium*, but was lower than for some other bacteria (Table 6.1). The majority of uptake systems have a high affinity for succinate, notable exceptions being *Salmonella typhimurium*, *Bacillus subtilis* and to a lesser extent, *Escherichia coli*. Although the "Km" values of the *Rhizobium* spp. are all fairly similar the "Vmax" values vary considerably, e.g. 0.3 to 7.0 nmol min^{-1} (mg cell protein)$^{-1}$ in bacteroids and 23.0 to 78.0 nmol min^{-1} (mg cell protein)$^{-1}$ in free-living cells. Low Vmax values for bacteroid preparations were also reported by Reibach and Streeter (1984), and may be due to difficulties in the preparation of material for assay, or more likely a reflection of the lack of growth with bacteroids.

It is probable that many of the rates shown in Table 6.1 are under-estimates, either due to the method used in sample preparation prior to quantification (during which substantial metabolism of the transported succinate to carbon dioxide may have occurred, especially with the "dry method"), or through the use of 1,4-[^{14}C]succinate

Table 6.1. Kinetics of succinate incorporation by bacteria.

Organism	Kinetic parameter		Reference
	Km (μM)	Vmax	
R. leguminosarum	2.6	78.0[a]	Finan *et al.*, (1981).
R. meliloti	5.3	23.9[a]	Engelke *et al.*, (1987).
B. japonicum	7.5	23.0[a]	San Francisco & Jacobsen (1985).
free-living			
bacteroid	10.1	0.7-3.0[a]	
Ps. putida	12.5	5.8[b]	Dubler *et al.*, (1984).
B. subtilis	100.0	20.0[b]	Ghei & Kay, (1973).
S. typhimurium	97.0	12.9[b]	Kay & Cameron, (1978).
E. coli	30.0	25.0[b]	Kay & Kornberg, (1971).

[a] ; nmol min^{-1} (mg cell protein)$^{-1}$.
[b] ; nmol min^{-1} (mg cell dry wt)$^{-1}$.

(which would transiently enhance this latter effect). The rates of [^{14}C]succinate incorporation measured for *A. caulinodans*, are therefore significantly more accurate.

In summary, the measured rates of [^{14}C]succinate incorporation for *A. caulinodans* do not provide definitive information on the kinetics of transport, although the Km for transport must be equal to or lower than the "Km" for incorporation ($\leq$ 20 μM). The results presented in Table 6.1, suggest that the Km for succinate transport in rhizobia is probably very low, which may be an important feature of bacteroid C_4-dicarboxylate metabolism.

6.2.2 The effect of metabolic inhibitors on the rate [^{14}C]succinate incorporation.

Potential inhibitors of succinate transport and metabolism were screened for their effect on [^{14}C]succinate incorporation by washed cells *A. caulinodans* .

The most potent inhibitor of [^{14}C]succinate incorporation tested was the uncoupling agent FCCP (Fig 6.5), which caused 50% inhibition of the incorporation rate at a concentration of 1.6 μM and caused complete inhibition (at 20 μM) of both [^{14}C]succinate incorporation and disappearance (Figs 6.3 & 6.4). It should be noted that very low amounts of radio-label were still detected in the cells under these conditions; however, the amounts did not increase with time and hence probably represented non-specific binding.

A variety of uncoupling agents (FCCP, CCCP and 2,4-DNP) have previously been reported to inhibit [^{14}C]succinate incorporation for a number of bacteria; *S. typhimurium* (Kay and Cameron, 1978) *B. subtilis* (Ghei and Kay, 1973), free-living cells and bacteroids of

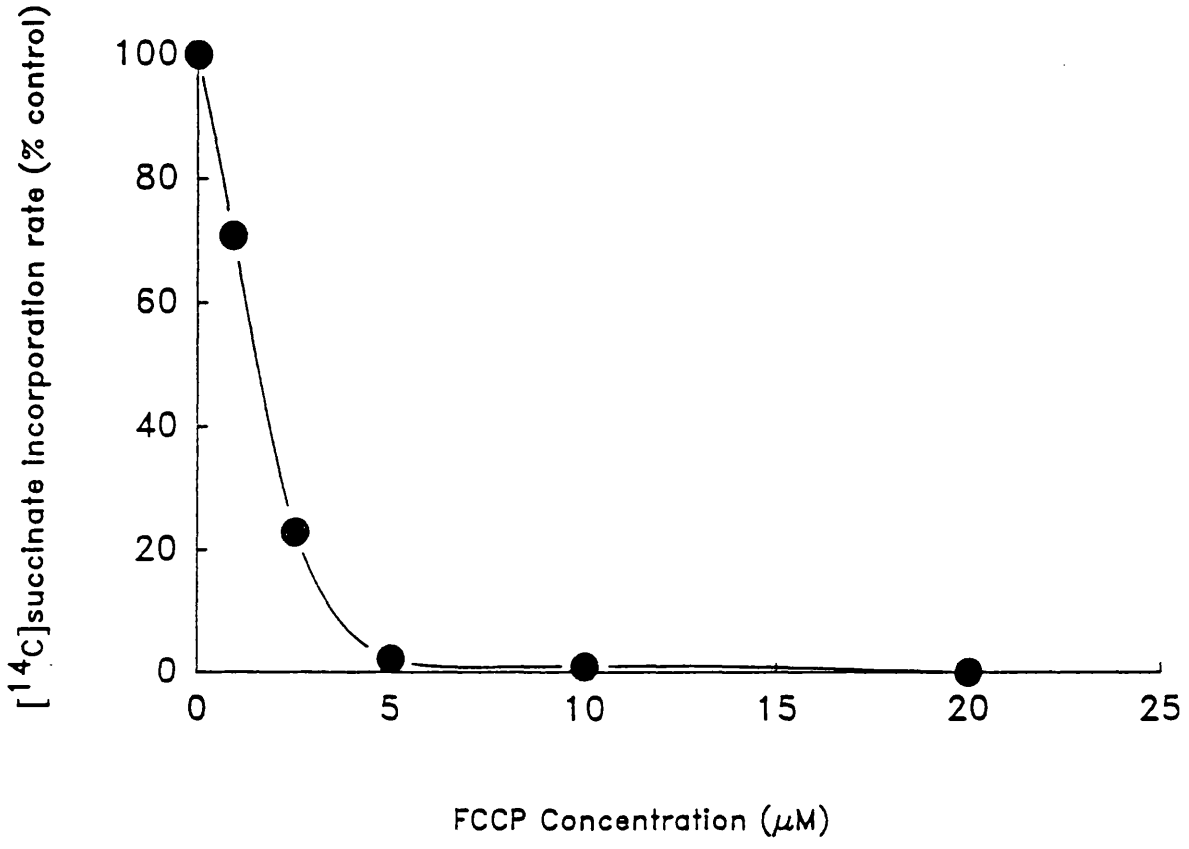


Figure 6.5. The effect of FCCP concentration on the rate of [^{14}C]succinate incorporation (250μM), by washed cells *A. caulinodans* grown under succinate limitation at D=0.03h^{-1}. [^{14}C]succinate incorporation was measured using the dry assay procedure as described in Materials and Methods. The uninhibited rate was 22.1 nmol min $^{-1}$ (mg cell dry wt)$^{-1}$.

R. leguminosarum (Glenn *et al.*, 1980), and free-living cells and bacteroids of *B. japonicum* (San Francisco and Jacobsen, 1985).

Potential sulphydryl reagents (1 mM) were tested against [^{14}C]succinate incorporation and succinate oxidation (Table 6.2). pCMB was the most effective inhibitor, causing 97.3% inhibition of [^{14}C]succinate incorporation and total inhibition of succinate oxidation. In contrast, pCMBS, the sulphonated derivative of pCMB, was considerably less effective, causing 19.8 and 12.1% inhibition respectively. The marked differences between the inhibitory effect of pCMB and pCMBS was presumably due to the presence of the large hydrophilic sulphonate group restricting access of the pCMBS into the cell and thus preventing inhibition of transport and/or metabolism.

The two other sulphydryl reagents tested, NEM and mersalyl acid ((O-[(3 [Hydroxymercuri]-2-methoxypropyl)-carbamoyl]-phenoxy-acetic acid), also inhibited [^{14}C]succinate incorporation and succinate oxidation to similar extents, albeit between those of pCMB and pCMBS.

The inhibitory nature of sulphydryl reagents on organic acid incorporation has been widely reported in the literature; Kay and Cameron (1978), reported inhibition of activity with NEM and pCMB on citrate incorporation for *S. typhimurium*. Ghei and Kay (1973), demonstrated inhibition of succinate incorporation with pCMB and NEM in *B. subtilis*. San Francisco and Jacobsen (1985), inhibited succinate incorporation in free-living cells of *B. japonicum* using 1mM NEM and Glenn *et al.* (1980), reported a 95% inhibition of succinate incorporation in *R. leguminosarum* also using 1mM NEM; the inhibition was approximately 7-fold greater than that reported for *B. japonicum* by San Francisco and Jacobsen (1985) at the same concentration and may reflect additional physiological differences between the transport/metabolism of [^{14}C]succinate between these different groups of rhizobia. Interestingly, *A. caulinodans* exhibited inhibition of succinate incorporation approximately mid-way between *B. japonicum*

Table 6.2. The effect of sulphydryl reagents (1mM), on the rate of [^{14}C]succinate incorporation and succinate oxidation by washed cells of *A. caulinodans* grown in continuous culture at D=0.1h^{-1}. Cells were pre-incubated with the sulphydryl reagent for 1 min prior to succinate addition. [^{14}C]succinate incorporation was measured using the dry assay procedure as described in Materials and Methods.

Sulphydryl Reagent	[^{14}C]succinate Incorporation Rate[a]	%	Succinate Oxidation Rate[b]	%
Control	29.8	100.0	280.0	100.0
pCMBS	23.9	80.2	246.0	87.9
Mersalyl acid	18.1	60.7	191.0	68.2
NEM	12.4	41.6	116.0	41.4
pCMB	0.8	2.7	0.0	0.0

[a] : nmol min^{-1} (mg cell dry wt)$^{-1}$

[b] : ng-atom O min^{-1} (mg cell dry wt)$^{-1}$

and *R. leguminosarum* at the same inhibitor concentration, which may reflect its apparent taxonomic position between the two classical groups of rhizobia (Dreyfus *et al.*, 1988).

The specificity of the succinate transport system of *A. caulinodans* was examined by measuring the effect of unlabelled organic acids on [^{14}C]succinate incorporation by washed cells prepared from cultures grown under succinate limitation at D=0.10h^{-1}. Equimolar lactate did not affect the rate of [^{14}C]succinate incorporation significantly (Table 6.3), whereas the C_4-dicarboxylates malate, fumarate and maleate inhibited [^{14}C]succinate incorporation by 70.4, 59.0 and 40.5% respectively. The simplest interpretation of these results is that succinate, fumarate, malate and maleate are transported *via* a common carrier. Inhibition of succinate incorporation by C_4-dicarboxylates in a variety of free-living *Rhizobium* spp. has been widely reported (Glenn *et al.*, 1980; Finan *et al.*, 1981; McAllister and Lepo 1983; San Francisco and Jacobsen 1985; Engelke *et al.*, 1987) and concluded that succinate, malate and fumarate were transported *via* a common system. Some differences in carrier-substrate affinity were noted; malate was generally the highest affinity substrate, except in *R. leguminosarum* (Glenn *et al.*, 1980), where it was replaced by fumarate. Although no Km values for the various dicarboxylates have been measured in *A. caulinodans*, the inhibition results described above are commensurate with the conclusion that malate is the preferred dicarboxylate for *A. caulinodans* (malate >fumarate >succinate >maleate). This conclusion should be treated with some caution however, since the competitive effects of the various substrates may not necessarily be confined to the transport level, but may be acting on metabolism.

Attempts to measure the pH changes which occurred during succinate transport and incorporation were made using the method of Henderson *et al.* (1977); washed cells were incubated under anaerobic conditions in 0.1 mM HEPES/KOH buffer (pH 7.0), adding 100 mM succinate (pH 7.0) and the change in pH was followed with a sensitive pH

Table 6.3. The effect of equimolar (250 μM) concentrations of unlabelled carbon substrates on the rate of [^{14}C]succinate incorporation into washed cells *A. caulinodans* grown under succinate limitation at D=0.1h^{-1}. [^{14}C]succinate incorporation was measured using the wet assay procedure as described in Materials and Methods.

Additions	[^{14}C]succinate Incorporation Rate (nmol min^{-1} [mg cell dry wt]$^{-1}$)	(%)
-	69.0	100.0
Lactate	68.0	98.6
Maleate	41.0	59.5
Fumarate	28.3	41.0
Malate	20.4	29.6

electrode. If the transport mechanism was linked to an inward H^+ translocation, then an alkaline pH change would be observed. However, the results were neither quantifiable nor consistently reproducible. The difficulties were attributed to very small, but significant, mis-matching of the pH of the reaction mix and the injected succinate. Although every reasonable effort was made to resolve this problem, the mis-matching was never totally eliminated and the experimental results remained inconsistent.

Attempts were therefore made to monitor pH changes *in vivo* of non pH-controlled batch cultures. These showed that the pH of the culture, the biomass concentration and rate of CO_2 production all increased as a function of time. Similarily, when the culture was maintained at pH 6.8 by the addition of 1M-HCl (Fig 6.6), the amount of HCl added increased in parallel with the cell density (measured as OD_{600}). Analysis of the culture supernatant for extracellular product using HPLC and plasma emission spectroscopy indicated that the succinate was metabolised to cells and carbon dioxide.

The growth of a closely-related organism *Agrobacterium radiobacter* on a similar minimal salts medium supplemented with glucose rather than succinate caused substantial acidification of the growth medium (presumably due to the dissociation of NH_4^+ to NH_3 prior to uptake of the ammonia and not due to transport since glucose is not taken up *via* an H^+/glucose symport; J.A. Greenwood and C.W. Jones, unpublished). The alkalinization of the *A. caulinodans* medium during growth on succinate was therefore strongly indicative of the presence of an H^+/succinate symport system for succinate transport.

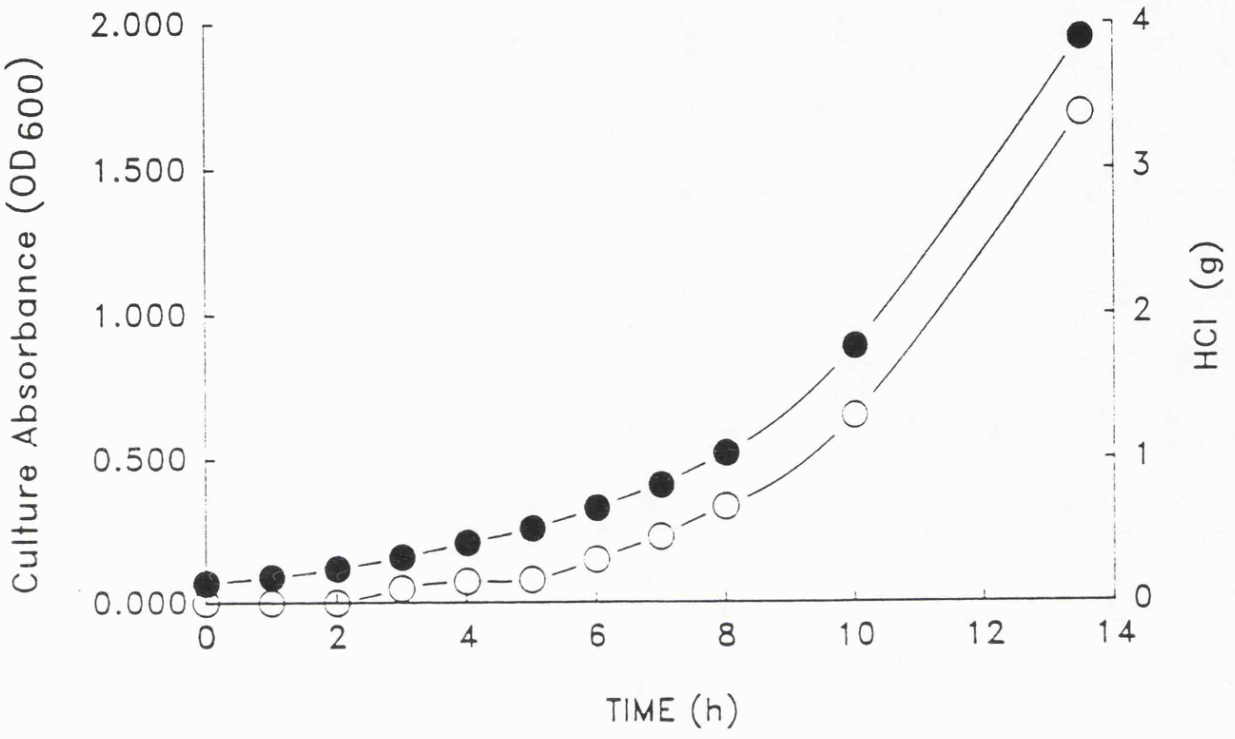


Figure 6.6. Addition of 1M-HCl to an exponential growing batch culture of *A. caulinodans* in order to maintain a setpoint of pH 6.8. The addition of 1M-HCl was measured using a tared electronic balance, and cell growth was measured spectrophotometrically as described in Materials and Methods. Culture Absorbance (OD_{600}), ● ; HCl ○ .

6.2.3 Incorporation and metabolism of [^{14}C]maleate in *A. caulinodans*.

In the absence of a sophisticated quench flow apparatus (see Lowe and Walmsley, 1985) accurate measurement of [^{14}C]succinate transport is extremely difficult due to the short period of net transport which is followed by a rapid rate of metabolism (transport being obscured after only a few seconds).

The accurate determination of solute transport rates and accumulation ratios can be achieved using two fundamentally different experimental approaches. Firstly, through the use of mutant organisms defective in metabolism (e.g. using succinate dehydrogenase minus mutants to measure succinate transport) and secondly, using non-metabolisable analogues of the solute under study. Both approaches have limitations. The use of sdh$^-$ cells for example, requires growth on alternative carbon sources which may perhaps adversely affect the system under study causing repression effects *etc*, and the use of analogues requires that they are transported in the same way as the "natural" solute and that they are truly non-metabolisable.

The use of mutants defective in C_4-dicarboxylate metabolism was not suitable for this type of physiological study with *A. caulinodans*, and therefore the use of a non-metabolisable analogue was adopted. A number of non-metabolisable succinate analogues have been reported, including malonate, maleate, bromosuccinate and fluorosuccinate. However, only maleate was readily available in a radio-labelled form and even that had to be prepared from [^{14}C]maleic anhydride (see Materials and Methods).

The rate of 2,3-[^{14}C]maleate incorporation by whole cells of *A. caulinodans* grown under succinate limitation was measured using the procedure described above for [^{14}C]succinate. The rate of [^{14}C]maleate incorporation was essentially unaffected by whether the

"dry assay" or the "wet assay" procedure was used, and in the latter case was very low compared to succinate (4 compared to 70 nmol min^{-1} [mg cell dry wt]$^{-1}$; Fig 6.7). The incorporation of [^{14}C]maleate was completely inhibited by the uncoupling agent FCCP (20 μM).

Maleate efflux experiments conducted over an extended time course, where accumulation of the radio-label lasted 21 min, showed efflux of radioactivity on the addition of FCCP (20 μM) (Fig 6.8). The rate of [^{14}C]maleate efflux was the same regardless whether the dry or wet assay procedure was used. The presented data suggest that the uptake of maleate proceeded *via* an active reversible process.

Hudman and Glenn (1980), reported efflux of [^{14}C]glucose on the addition of azide to pre-treated cells of *R. leguminosarum*, in a similar manner to that described above for FCCP induced efflux of [^{14}C]maleate incorporation. However, azide is an inhibitor of cytochrome oxidase activity and not an uncoupling agent. Ghei and Kay (1973), working with *B. subtilis* also reported dicarboxylate efflux on the addition of CCCP to pre-incubated cells, but with a $t_{\frac{1}{2}}$ of approximately 30 min.

Succinate (80 μM) inhibited the incorporation of [^{14}C]maleate (250 μM), by 50% (Fig 6.9). It should be noted however, that 1mM succinate failed to abolish maleate incorporation completely, the reasons for which are unclear.

[^{14}C]succinate (250 μM) incorporation was also inhibited by unlabelled maleate (Fig 6.10) although the I_{50} of maleate was much higher (350 μM) and probably reflected lower affinity of the transport system for maleate. Ghei and Kay (1973), also reported lower K_i values for maleate inhibition of succinate incorporation compared to malate or fumarate for *B. subtilis*. However, Dubler *et al.* (1984), reported that succinate incorporation for *Ps. putida* was unaffected by the presence of maleate, highlighting the differences that exist between different bacterial species.

Unlabelled succinate, malate and fumarate all inhibited

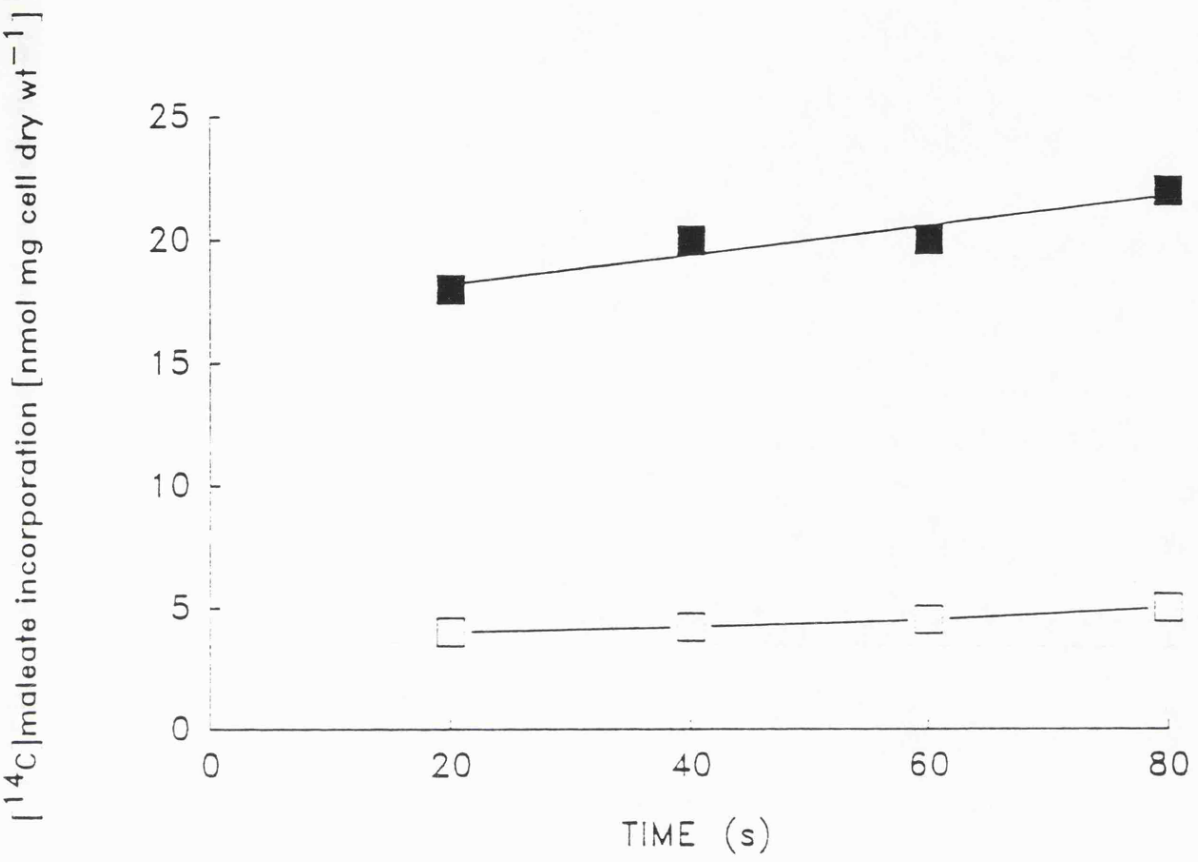


Figure 6.7. Incorporation of [^{14}C]maleate into washed cells of *A. caulinodans* grown under succinate limitation at D=0.10h^{-1}. [^{14}C]maleate incorporation was measured using the wet assay procedure as described in Materials and Methods, and occurred at a rate of 4.0 nmol min^{-1} (mg cell dry wt)$^{-1}$. Maleate, ■ ; Maleate + FCCP (20μM), □ .

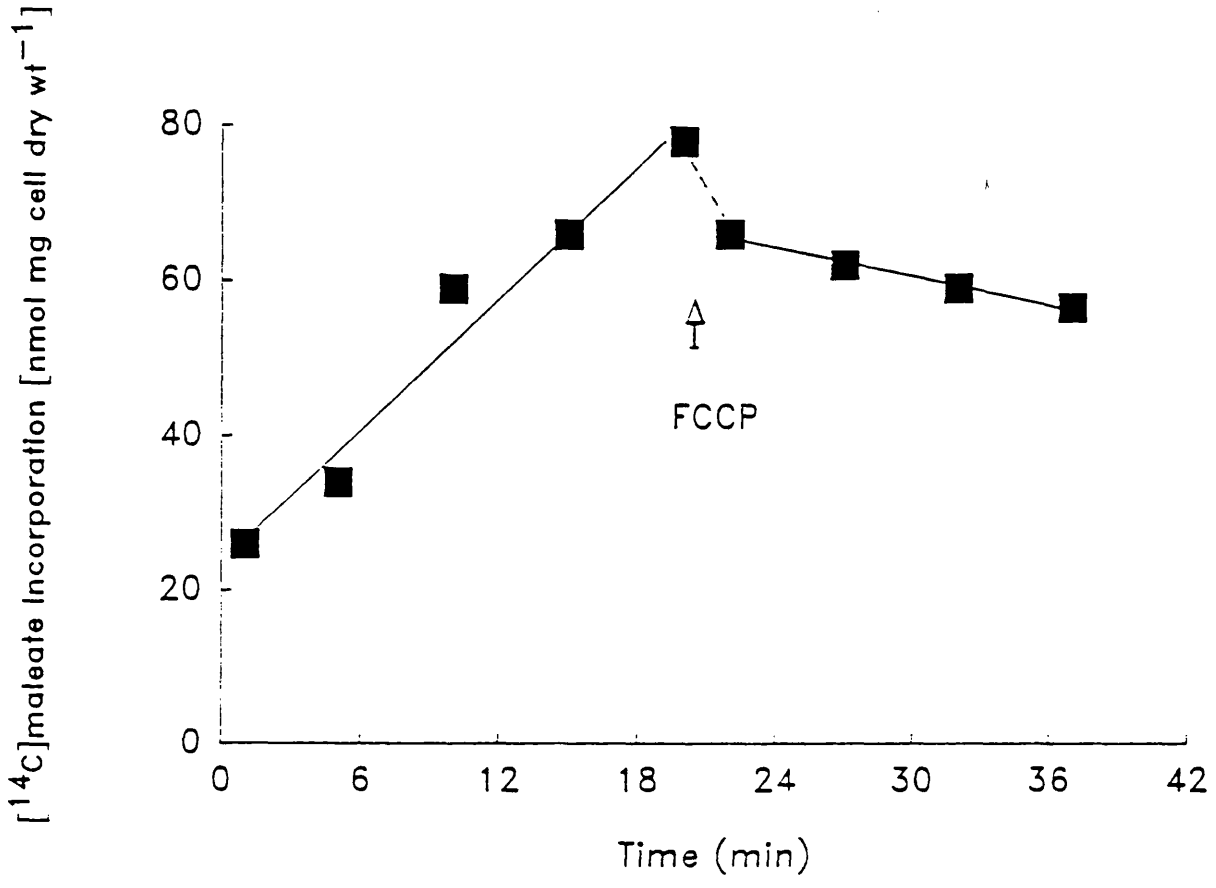


Figure 6.8. Efflux of [^{14}C]maleate from washed cells of *A. caulinodans* following addition of FCCP (20μM) to the reaction mix. Cells were grown under succinate limitation in continuous culture at D=0.1h^{-1}. [^{14}C]maleate incorporation occurred at a rate of 2.9 nmol min^{-1} (mg cell dry wt)$^{-1}$, and the initial efflux rate (dashed line) was at least 14.9 nmol min^{-1} (mg cell dry wt)$^{-1}$. [^{14}C]maleate incorporation and efflux was measured using the wet assay procedure as described in Materials and Methods.

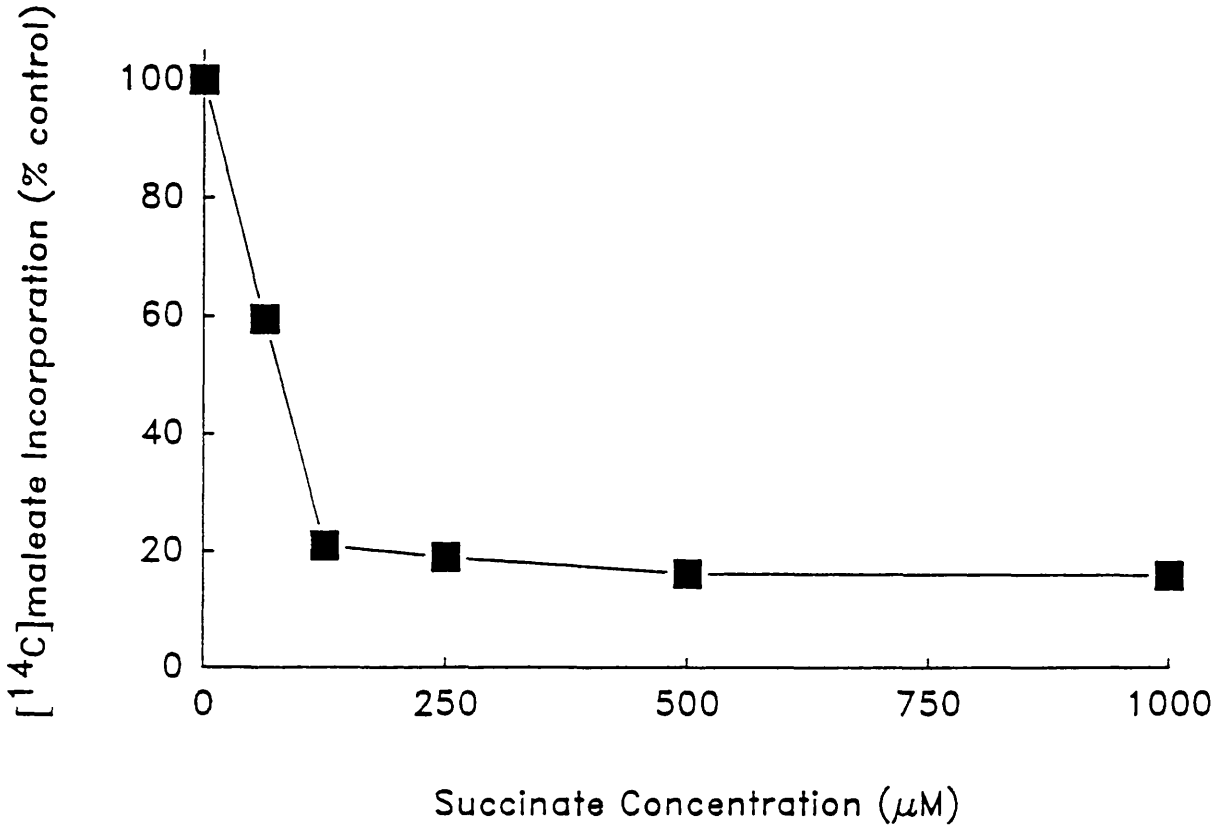


Figure 6.9. The effect of succinate concentration on the incorporation of [^{14}C]maleate (250 μM) by washed cells *A. caulinodans* grown under succinate limitation at D=0.065h^{-1}. Total [^{14}C]maleate incorporation was measured after 60s since the rate of incorporation was too slow to yield accurate results. The unlabelled succinate was added at the same time as the [^{14}C]maleate and incorporation measured using the wet assay procedure as described in Materials and Methods. The uninhibited [^{14}C]maleate incorporated after 60s was 14.6 nmol (mg cell dry wt)$^{-1}$.

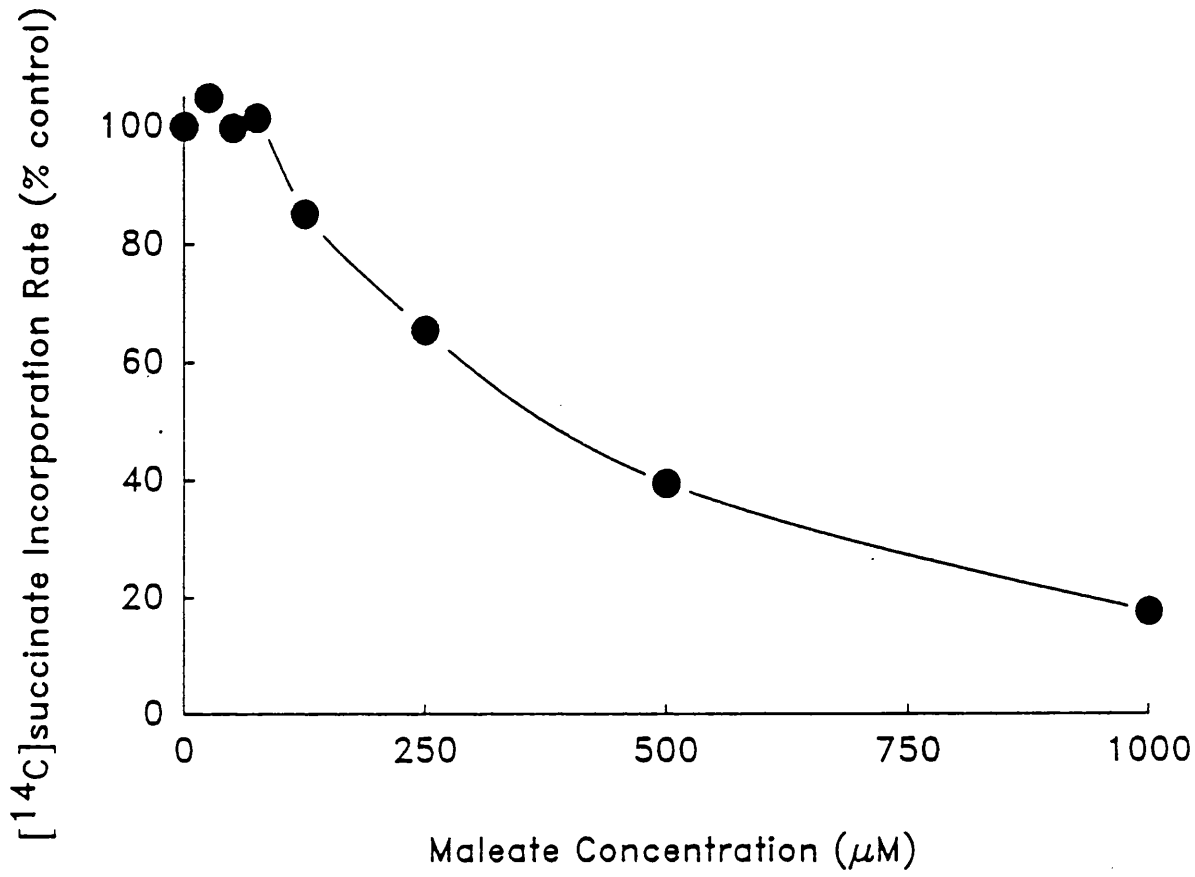


Figure 6.10. The effect of maleate concentration on the incorporation of [^{14}C]succinate (250μM) into washed cells of *A. caulinodans* grown under succinate limitation at D=0.065h^{-1}. [^{14}C]succinate incorporation was measured using the wet assay procedure as described in Materials and Methods. The unlabelled maleate was added at the same time as the [^{14}C]succinate. The uninhibited [^{14}C]succinate incorporation rate was 57.8 nmol (mg cell dry wt)$^{-1}$.

[^{14}C]maleate incorporation by approximately 70% when present at equimolar concentrations (Table 6.4), suggesting that the affinity of the C_4-dicarboxylate transport system was lower for maleate than for the other substrates. Lactate also inhibited [^{14}C]maleate incorporation by 14%, the degree of inhibition was higher than that observed for [^{14}C]succinate incorporation. The interpretation of this observation is unclear.

Paper chromatography of cell extracts of *A. caulinodans* prepared from cells which had been incubated with [^{14}C]succinate or [^{14}C]maleate for 10s, showed that succinate was metabolised to at least two major products (Fig 6.11*a*). The compound of R_F value 0.50-0.63 co-chromatographed with the succinate standard (Fig 6.11*b*), and arose from one spot on the autoradiogram that was bisected during preparation of the chromatogram strips. In contrast [^{14}C]maleate was metabolised to probably only one minor product during this period (Figs 6.12*a* & 6.12*b*), and the unmetabolised maleate accounted for approximately 70% of the accumulated label.

Both [^{14}C]succinate and [^{14}C]maleate gave rise to a product of R_F value of 0.27. Neither this product, or the product of R_F value 0.80, was identified.

The measured accumulation ratio for maleate into cells of *A. caulinodans* was 19.8 ± 5.2 (n=4). The accumulation ratio was calculated from the amount of [^{14}C]carbon extracted from washed cell preparations incubated with [^{14}C]maleate for 10s used in the paper chromatography experiments described above, quench corrected and referenced to the external [^{14}C]maleate concentration, using an intracelluar volume of 0.73 μl mg^{-1} cell dry wt. The accumulation ratio of 19.8 was commensurate with the presence of an H^+ symport rather than an ATP dependent (binding protein) system since the respective accumulation ratios can be calculated to be approximately 40 and 1000 respectively (see Henderson, 1986).

Table 6.4. The effect of equimolar (250 μM), concentrations of unlabelled carbon substrates on the incorporation of [^{14}C]maleate after 60s incubation into washed cells *A. caulinodans* grown under succinate-limitation at D=0.1h^{-1}. [^{14}C]maleate incorporation was measured using the wet assay procedure as described in Materials and Methods.

Carbon Substrate	[^{14}C]maleate Accumulation (nmol [mg cell dry wt]$^{-1}$)	(%)
-	14.3	100.0
Lactate	12.3	86.0
Succinate	4.4	30.8
Fumarate	4.3	30.1
Malate	4.3	30.1

Figure 6.11*a*. Histogram of radioactivity in chromatographed cell extracts from *A. caulinodans*. The organism was grown under succinate limitation at $D=0.10h^{-1}$. Washed cells were incubated for 10s with [^{14}C]succinate prior to extraction into hot water as described in Materials and Methods. Component 1, R_F value, 0.27; Component 2, R_F value, 0.50-0.63; Component 3, R_F value, 0.80.

Figure 6.11*b*. [^{14}C]succinate control, R_F value, 0.50.

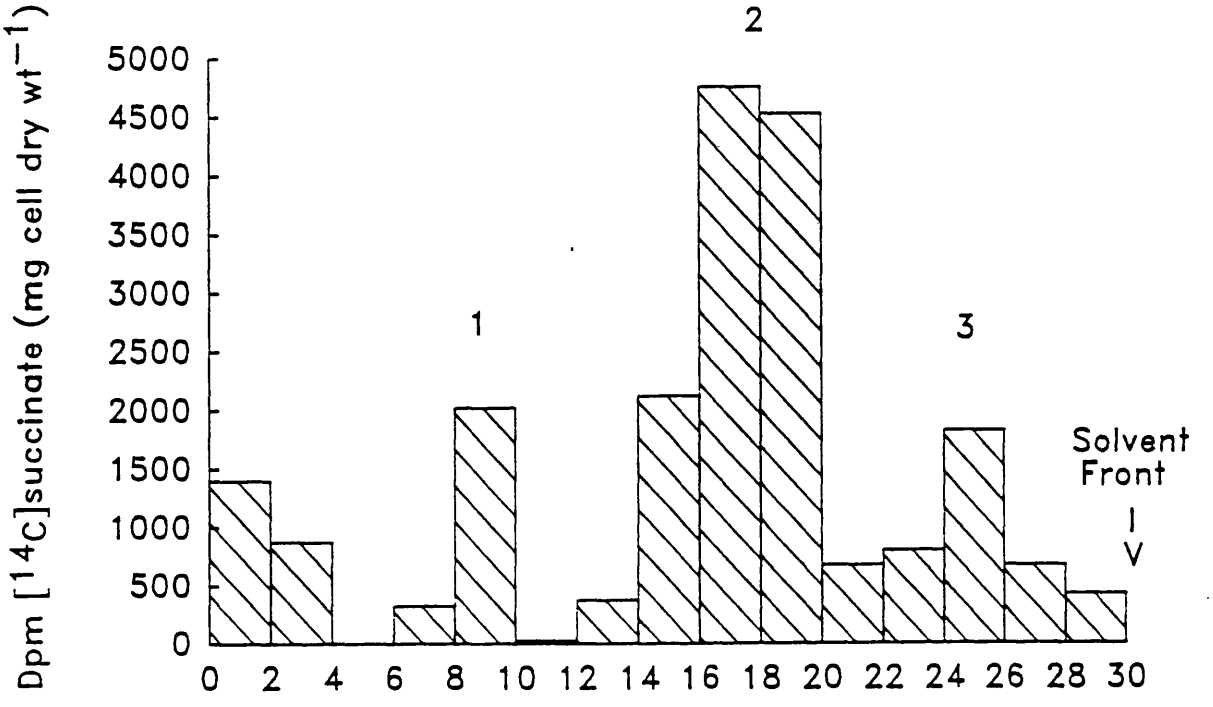


Figure 6.11a

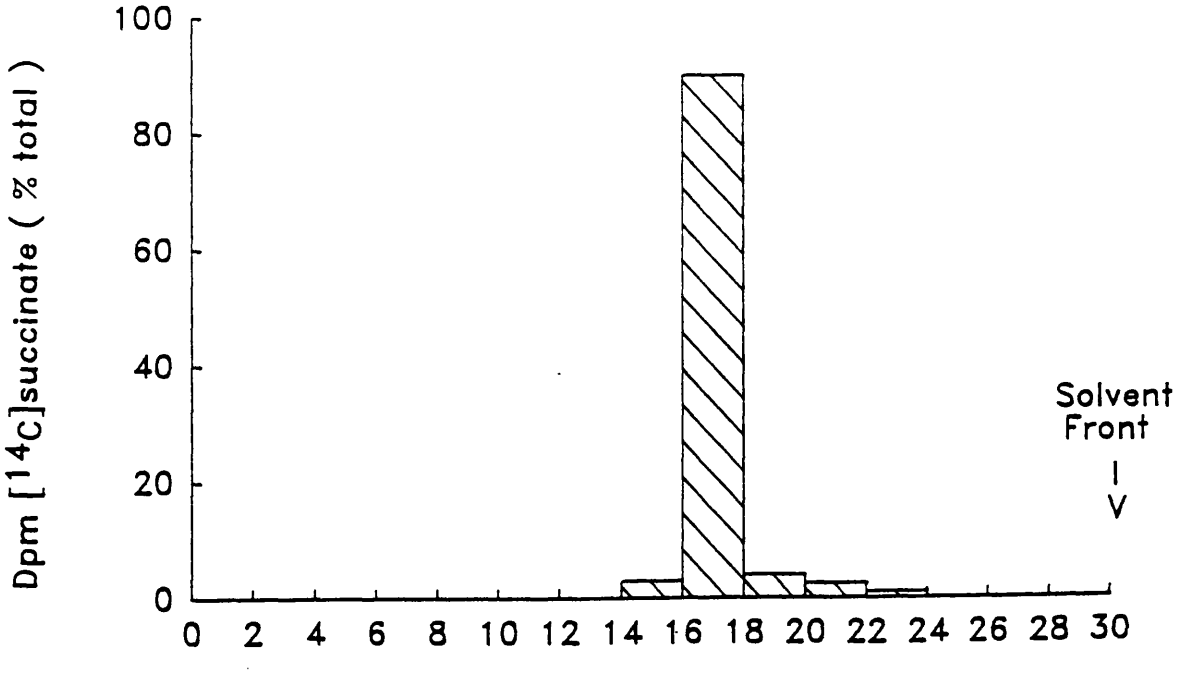


Figure 6.11b

Figure 6.12*a*. Histogram of radioactivity in chromatographed cell extracts from *A. caulinodans*. The organism was grown under succinate limitation at $D=0.10h^{-1}$. Washed cells were incubated for 10s with [^{14}C]maleate prior to extraction into hot water as described in Materials and Methods. Component 1, R_F value, 0.27; Component 2, R_F value, 0.50-0.63.

Figure 6.12*b*. [^{14}C]maleate control, R_F value, 0.50.

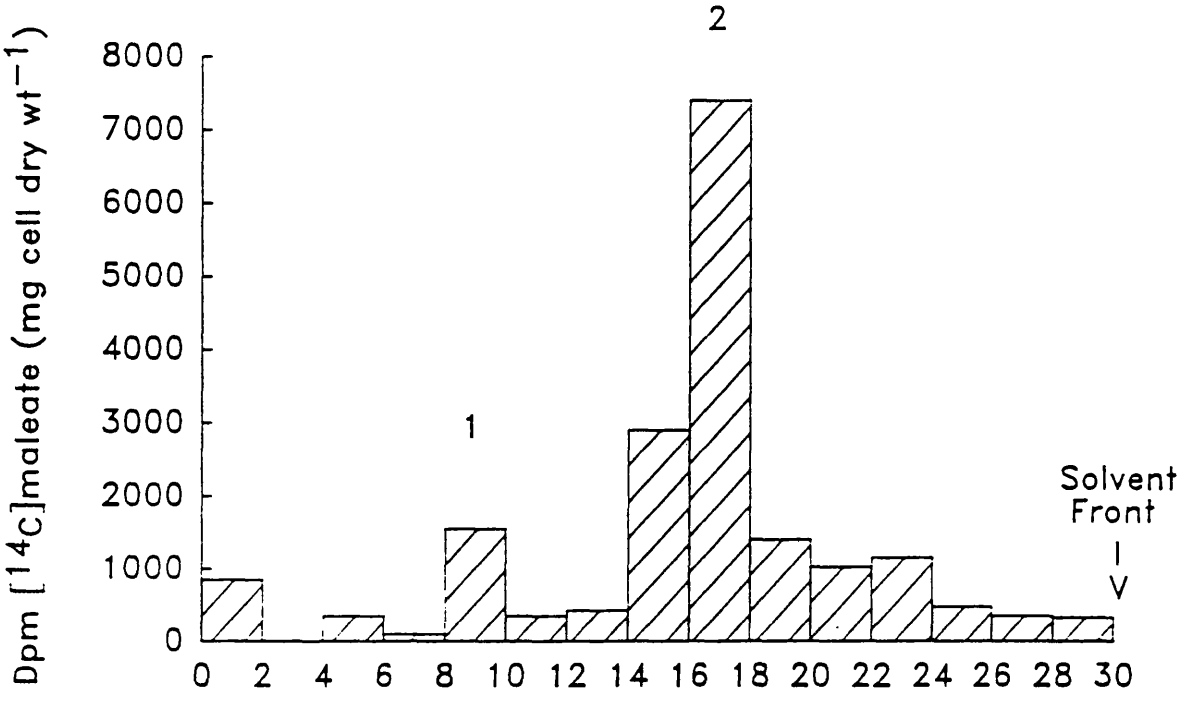


Figure 6.12a

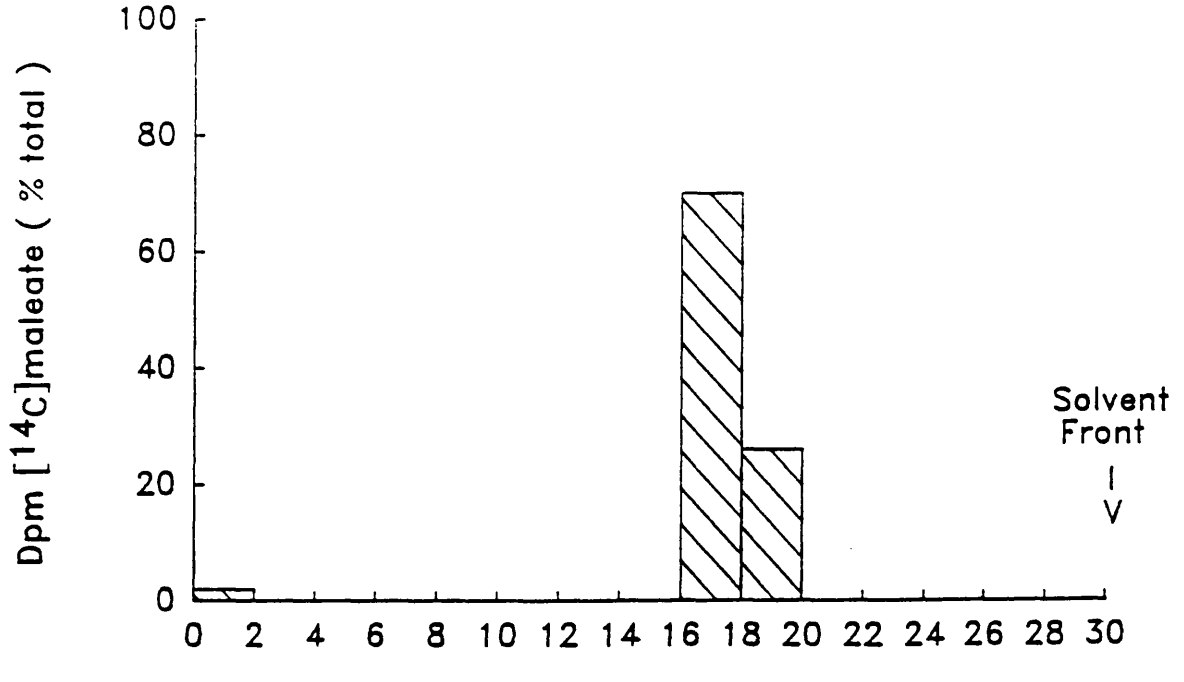


Figure 6.12b

6.2.4 Transport of [^{14}C]succinate and [^{14}C]maleate.

The rates of [^{14}C]succinate and [^{14}C]maleate transport were measured discontinuously over the first 5s following the addition of substrate. As the assay could not be completed in less than 5s comparison of the amount of substrate taken up after 5s to that of the FCCP-treated cells at zero time allowed the calculation of the *minimum* transport rates for succinate and maleate. The rates for cells grown under succinate-limitation (D=0.10 h^{-1}) were 245 and 144 nmol min^{-1} (mg cell dry wt)$^{-1}$ for succinate and maleate respectively (Figs 6.13 & 6.14). It should be noted, of course that the actual transport rates may obviously be faster than these rates depending upon how much less than 5s it takes for the substrate to reach a steady-state concentration within the cell, and hence for the rates of incorporation to be determined by the rate of metabolism.

The rates of [^{14}C]succinate and [^{14}C]maleate incorporation were 78 and 4 nmol min^{-1} (mg cell dry wt)$^{-1}$ respectively measured between 5 and 15s. These much lower rates indicate that the rate of transport had already become limited by the rate of metabolism.

Dicarboxylate transport in *Rhizobium* is poorly documented compared to dicarboxylate incorporation. Published time courses are frequently too long (minutes rather than seconds), filtered cells are often dried for long periods prior to scintillation counting and corrections for non-specific substrate binding are rarely made. Furthermore, rates of dicarboxylate incorporation from published graphical data are frequently interpolated back to the origin which suggests that incorporation and transport are synonymous (especially where Km values are presented) (Glenn *et al.*, 1980; Saroso *et al.*, 1984; Bolton *et al.*, 1986; Birkenhead *et al.*, 1988). Careful analysis of succinate "uptake" data from San Francisco and Jacobsen (1985) and by McKay *et al.* (1988) confirm that an initial fast rate

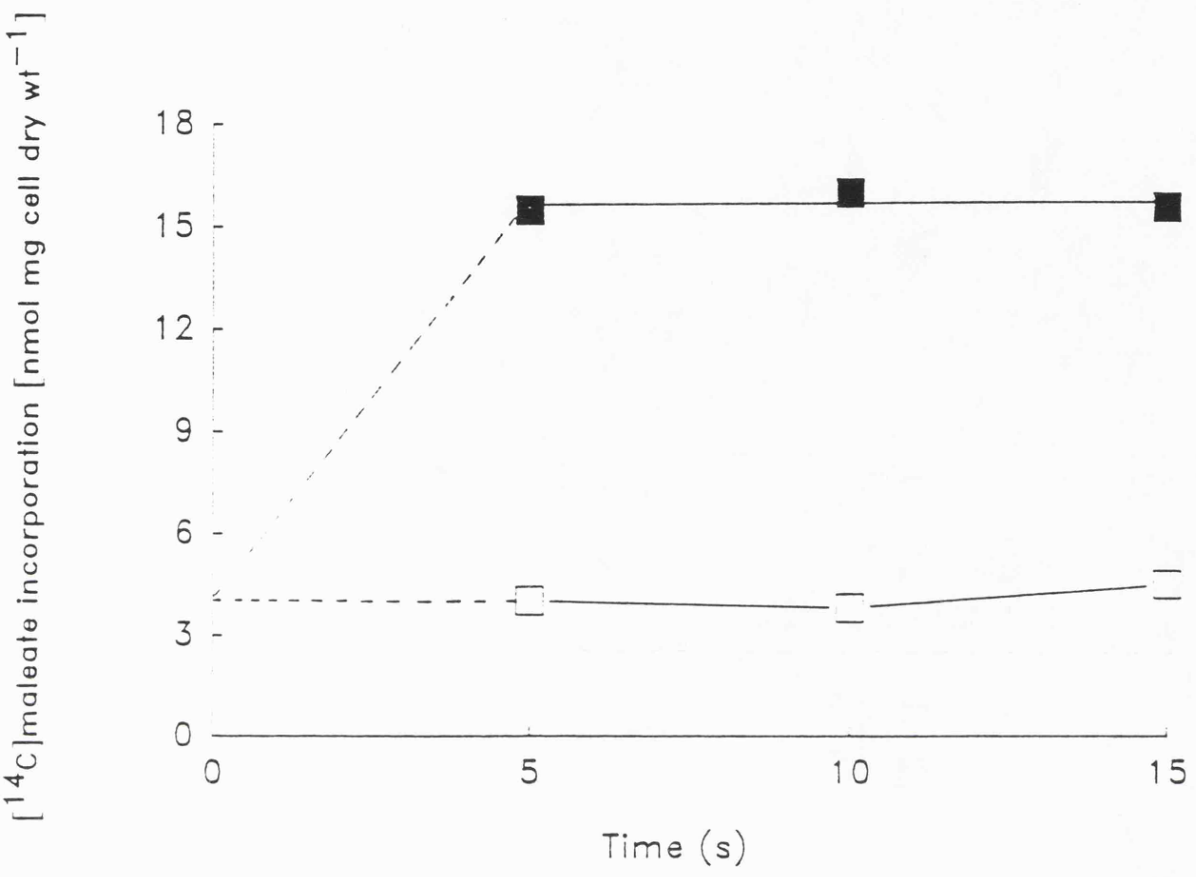


Figure 6.13. Incorporation of [^{14}C]maleate by washed cells of *A. caulinodans* grown under succinate limitation at D=0.1h^{-1}. [^{14}C]maleate incorporation was measured using the wet assay procedure as described in Materials and Methods. The rate of maleate transport was calculated from the rate of maleate incorporation over the first 5s corrected for non-specific binding, and was at least 144 nmol min^{-1} (mg cell dry wt)$^{-1}$. Maleate, ■ ; Maleate + FCCP (20μM), □ .

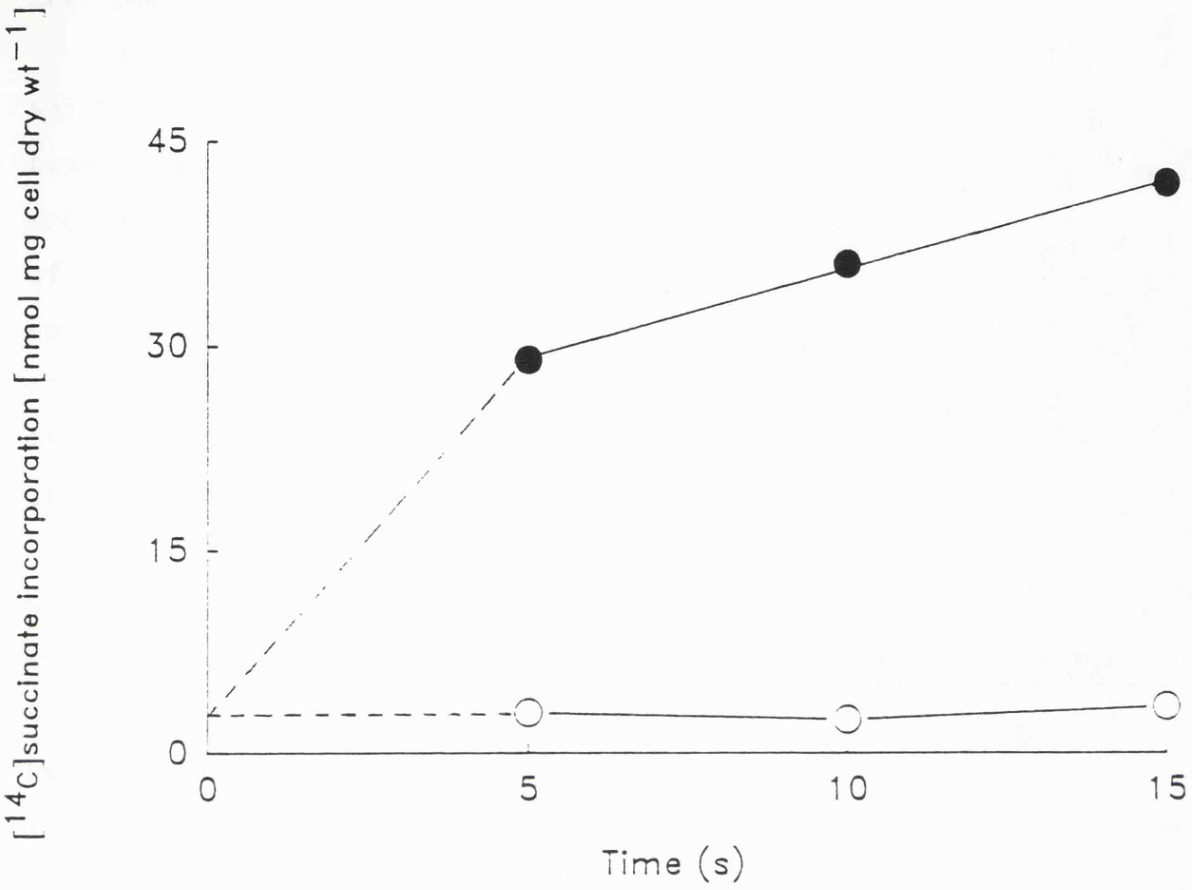


Figure 6.14. Incorporation of [^{14}C]succinate by washed cells of *A. caulinodans* grown under succinate limitation at D=0.1h^{-1}. [^{14}C]succinate incorporation was measured using the wet assay procedure as described in Materials and Methods. The rate of succinate transport was calculated from the rate of succinate incorporation over the first 5s corrected for non-specific binding, and was at least 245 nmol min^{-1} (mg cell dry wt)$^{-1}$. Succinate, ● ; Succinate + FCCP (20μM), ○ .

of transport occurred similar to that described above for *A. caulinodans*, since the extrapolated data intercepted the ordinate axis at a value significantly greater than that obtained for CCCP-treated or dct$^-$ cells. The original data points were, however, plotted such that the uptake rate passed through the origin.

An alternative approach to measuring dicarboxylate transport which has been applied to *Rhizobium* is to use succinate dehydrogenase mutants (sdh$^-$). Unfortunately, genuine sdh$^-$ mutants have proved difficult to obtain. Finan *et al.* (1981) generated a mutant of *R. leguminosarum* using NTG that grew very slowly on succinate compared to malate or fumarate, but still incorporated [^{14}C]succinate at half the wild type rate (36 *cf* 78 nmol min^{-1}[mg cell protein]$^{-1}$), suggesting that the lesion may not necessarily be associated with the sdh.

Ghei and Kay (1973) generated an sdh$^-$ mutant of *B. subtilis* which incorporated [^{14}C]succinate but only at a rate of 1.5 nmol min^{-1}(mg cell dry wt)$^{-1}$. This very low rate of incorporation may have resulted from the extremely low succinate concentration (10 μM) used in the assay procedure compared with the relatively high Km (100 μM), for transport. Alternatively, the system may have been energy-limited, such that the accumulated succinate could not be metabolised or endogenous reserves were too low to provide sufficient energy to activate the system fully.

The very fast rate of C_4-dicarboxylate transport observed with washed cells of *A. caulinodans* grown in continuous culture under succinate limitation suggests that the cells have adapted phenotypically to an environment with low substrate availability. This is a common response of first enzymes in metabolic pathways such as solute transport systems and periplasmic dehydrogenases (Harder and Dijkhuizen, 1983).

The effect of growth under different nutrient limitations on the rates of [^{14}C]succinate and [^{14}C]maleate transport and incorporation was studied by growing *A. caulinodans* in continuous culture

under succinate, sulphate or oxygen limitation ($D=0.10h^{-1}$). The rates of [^{14}C]succinate and [^{14}C]maleate transport were apparently much higher in cells grown under succinate limitation than under sulphate or oxygen limitation, the rate of maleate transport being approximately 60-70% of the rate with succinate in each case. In contrast the rates of [^{14}C]succinate incorporation were similar in the three types of cells; the rates of [^{14}C]maleate incorporation were higher in cells grown under succinate limitation than under sulphate or oxygen limitation but the rates were extremely low in all types of cells (Table 6.5). *In vivo* $q_{succinate}$ values for cells grown under comparable conditions of succinate, sulphate and oxygen limitation, were 40.2, 42.7 and 41.8 nmol min^{-1} (mg cell dry wt)$^{-1}$ respectively (see 3.2.6), i.e. very similar to each other and slightly lower than the *in vitro* values.

The results obtained for [^{14}C]succinate and [^{14}C]maleate transport strongly suggest derepression of the dicarboxylate transport system under conditions of succinate limitation, and are consistent with the presence of a low steady-state concentration of succinate in the growth medium during growth at low dilution rates as predicted by the Monod equation. Under these conditions the activity of the transport system increases to compensate for the low substrate concentration.

6.2.5 Physiological regulation of succinate metabolism.

The ability of different carbon sources to act as either inducers or repressors of [^{14}C]succinate incorporation by *A. caulinodans* was studied using washed cells prepared from batch culture.

Cells grown with either succinate, malate or fumarate as the

Table 6.5. The transport and incorporation rates of [^{14}C]succinate and [^{14}C]maleate by washed cells of *A. caulinodans* grown in continuous culture (D=0.1h^{-1}) under succinate, oxygen and sulphate limitation. Rates were measured using the wet assay procedure as described in Materials and Methods. Transport rates are minimum values and are the mean of two independent determinations.

Activity	Growth-Limiting Nutrient		
	Succinate	Oxygen	Sulphate
	(nmol min^{-1} [mg cell dry wt]$^{-1}$)		
Succinate Transport	245	102	131
Succinate Incorporation	75 ± 5 (4)	53 ± 5 (3)	77 ± 8 (3)
qSuccinate *In situ*	40	42	43
Maleate Transport	144	62	93
Maleate Incorporation	4 ± 1 (3)	1 ± 0 (3)	1 ± 0 (3)

sole carbon source exhibited [^{14}C]succinate incorporation rates that were high and not significantly different from each other (Table 6.6). However, cells cultured with either lactate or gluconate as sole carbon source exhibited substantially lower [^{14}C]succinate incorporation rates. Succinate respiration rates exhibited a similar pattern to the succinate incorporation rates. Cells grown on succinate plus lactate (50mM each), exhibited [^{14}C]succinate incorporation and succinate respiration rates which were similar to those of cells grown on succinate alone. These results indicated that the enzyme(s) responsible for determining the rate of dicarboxylate incorporation were induced by succinate, fumarate and malate and possibly maleate (albeit weakly), but not by any of the monocarboxylates tested. As the sdh activity did not change significantly following growth on lactate it is possible that the induction of dicarboxylate incorporation was due to induction of the dicarboxylate transport system.

Previous work with *R. meliloti* and cowpea-type *Rhizobia* (Saroso *et al.*, 1984, Engelke *et al.*, 1987), showed that C_4-dicarboxylate incorporation was inducible by succinate, and the system was not present in cells cultured on glucose as sole carbon source. Inducible C_4-dicarboxylate "transport" systems have been reported for *R. trifolii* (Ronson *et al.*, 1981) *R. leguminosarum* (Finan *et al.*, 1981), and *B. japonicum* (McAllister and Lepo, 1983), as well as for *Pseudomonas putida* (Dubler *et al.*, 1974), and *B. subtilis* (Ghei and Kay 1973). Constitutive C_4-dicarboxylate "transport" systems have been reported for a different strain of *R. leguminosarum* (Glenn *et al.*, 1980) and two *Bradyrhizobium* spp. (San Francisco and Jacobsen 1985). Equimolar mixtures of succinate and glucose also induced succinate incorporation in cowpea-type *Rhizobia* (Saroso *et al.*, 1984), suggesting that glucose (like lactate in *A. caulinodans*) was not acting as a repressor. In contrast, partial repression of succinate incorporation by glucose was reported for *R. trifolii* by Ronson *et al.* (1981).

Table 6.6. The effect of different growth substrates on the rate of [^{14}C]succinate incorporation, succcinate respiration and succinate dehydrogenase activity in washed cells or broken cells of *A. caulinodans*. Cells were grown in batch culture with 2.4 gl^{-1} carbon substrate. Enzyme activities were measured as described in Materials and Methods. The mean value (2-4 determinations) for the rate of [^{14}C]succinate incorporation using the wet assay procedure was 48.2 nmol min^{-1} (mg cell dry wt)$^{-1}$, for succinate respiration was 124 ng atom O min^{-1} (mg cell dry wt)$^{-1}$, and for succinate dehydrogenase activity was 34.6 nmol min^{-1} (mg cell dry wt)$^{-1}$. ND, not determined.

Growth Substrate	[^{14}C]succinate Incorporation Rate (% control)	Succinate Respiration Rate (% control)	Succinate Dehydrogenase Activity (% control)
Succinate	100.0	100.0	100.0
Fumarate	90.0	117.7	ND
Malate	94.7	95.9	ND
Lactate	32.7	36.0	107.2
Gluconate	16.5	28.8	ND
Succinate + lactate	108.5	100.2	ND
Succinate + maleate	99.0	ND	89.6
Lactate + maleate	41.3	ND	81.0

The ability of maleate to act as an inducer of C_4-dicarboxylate metabolism was also investigated (Table 6.6). Cells incubated on maleate (50mM), as the sole carbon source failed to grow, although radio-labelling studies showed that it was slowly metabolised (see section 6.2.3). Incubation of cells with lactate and maleate either in equimolar concentrations (50mM) or with the maleate concentration decreased to 5mM, did not significantly increase the rate of [^{14}C]succinate incorporation, which remained well below that of cells grown on succinate or succinate plus maleate. Incubation of cells on lactate followed by incubation of the same cells with 50mM maleate also failed to increase [^{14}C]succinate incorporation. These results indicated that maleate did not act as an inducer of succinate incorporation in *A. caulinodans*. Maleate as an inducer of C_4-dicarboxylate incorporation has been reported by Kay (1971) for *E. coli*, and as a partial inducer for *Azotobacter vinelandii* (Reuser and Postma 1973), however, few other documented reports exist. The inability of maleate to act as an effective inducer of succinate incorporation for *A. caulinodans* is unclear considering that it is metabolised to a limited extent, suggesting that the first product of metabolism may not be fumarate (an effective inducer), or that the concentration of fumarate produced is too low.

In contrast to succinate incorporation, succinate dehydrogenase activity was found to be independent of the carbon source, indicating that the enzyme was probably synthesized constitutively. This conclusion is perhaps not surprising since succinate dehydrogenase is an integral component of the TCA cycle.

The rates of [^{14}C]succinate incorporation and succinate dehydrogenase activity were measured in washed cells of *A. caulinodans* grown under succinate limitation in continuous culture at different dilution rates (Table 6.7). [^{14}C]succinate incorporation increased with increasing dilution rate. Comparison of the *in vitro* and *in vivo* $q_{succinate}$ values obtained over the same range of dilution rates (see 3.2.6), showed that the *in vitro* rates were always higher

Table 6.7. The effect of dilution rate on the rate of [^{14}C]succinate incorporation and succinate dehydrogenase activity (Sdh) of washed and broken cells of *A. caulinodans* grown under succinate limitation in continuous culture.

Dilution Rate (h^{-1})	[^{14}C]succinate Incorporation Rate	Sdh Activity	*In situ* qSuccinate
	(nmol min^{-1} [mg cell dry wt]$^{-1}$)		
0.030	40.0 ± 4.5 (3)	22.54 ± 0.52 (3)	11.8
0.065	61.9 ± 1.7 (3)	28.00 ± 2.11 (3)	25.8
0.100	74.9 ± 5.1 (4)	27.90 ± 1.22 (3)	40.2
0.140	99.5 ± 6.5 (3)	33.90 ± 1.10 (3)	58.3

by a factor of approximately 2.5. This difference probably reflected the conditions of excess substrate employed *in vitro* compared with the low steady-state concentration of succinate *in vivo* (Monod 1942).

Succinate dehydrogenase activity also increased with dilution rate, from 22.5 to 33.9 nmol min^{-1} (mg cell dry wt)$^{-1}$. It should be noted however, that the succinate dehydrogenase activity did not satisfy the *in vivo* $q_{succinate}$ at dilution rates above $0.065h^{-1}$, despite taking all reasonable precautions to ensure accurate measurement of maximum sdh activity. This failure to correlate *in vitro* enzyme and *in vivo* activities highlights the possible dangers of extrapolating *in vitro* studies to the physiological level without careful analysis of the data. The latter is even more acute when performing pathway flux anaylses according to the methods of Kell and Westerhoff (1986), and Holms (1986), which place great emphasis on measured enzyme rates.

6.3 Discussion

In numerous studies the requirement for a functional C_4-dicarboxylate transport system to support symbiotic nitrogen fixation has been highlighted, and mutations that eliminate C_4-dicarboxylate incorporation functions have been shown to lead to an ineffective symbiosis (Ronson *et al.*, 1981; Finan *et al.*, 1983; Bolton *et al.*, 1986). The kinetics of C_4-dicarboxylate incorporation have also been studied in a number of rhizobia including bacteroid preparations (Glenn *et al.*, 1980; Arwas *et al.*, 1985), however, frequently these earlier studies yielded "transport" or metabolism rates considerably lower than could support the actual growth rate of the organism. The

frequent confusion of transport and metabolism and the low incorporation rates often stem from the use of 1,4-[^{14}C]succinate (Bolton *et al.*, 1986; McKay *et al.*, 1988; Birkenhead *et al.*, 1988), where the [^{14}C]atom is cleaved sooner to form CO_2 following the initial decarboxylation, and through the use of assay procedures that allow continued metabolism of the radio-label to [^{14}C]CO_2 during the quantification stage (Finan *et al.*, 1981; San Francisco and Jacobsen, 1985; Birkenhead *et al.*, 1988).

The values of C_4-dicarboxylate incorporation presented in this thesis for *A. caulinodans* are significantly higher than for many other rhizobia reported in the literature, but were found to be equal to or greater than the *in vivo* $q_{succinate}$ values obtained from measurements of molar growth yields. These observations clearly show that the *in vitro* incorporation rates using [^{14}C]succinate were accurate reflections of the *in vivo* activity, and were achieved through the use of 2,3-[^{14}C]succinate, coupled with a rapid cessation of metabolism prior to quantification.

The incorporation of C_4-dicarboxylates by *A. caulinodans* was clearly an active process since the energization of transport was markedly inhibited by agents that uncoupled respiration from ATP synthesis e.g. FCCP. The efflux of [^{14}C]maleate from washed cell preparations following the addition of FCCP showed that there was a positive net accumulation of [^{14}C]maleate against the concentration gradient, as also evidenced experimentally by an accumulation ratio of approximately 20, and the process was reversible.

The C_4-dicarboxylate system of *A. caulinodans* was almost certainly an H^+/symport mechanism and not a binding protein dependent system as proposed for *B. japonicum* by San Francisco and Jacobsen (1985), as shown by the low accumulation ratio, medium alkalization studies and indirectly through FCCP inhibition studies (uncoupling agents render the lipid membrane permeable to protons, and this has become a criterion for the identification of proton linked mechanisms; see Harold, 1972, 1977; Hamilton, 1975).

The transporter was probably common for succinate, fumarate, malate and maleate, and induced by all C_4-dicarboxylates tested (or products of their metabolism) with the exception of maleate. The inducible nature of the transporter is in close agreement with published reports for other rhizobia with the exception of a constitutive system reported for *B. japonicum* (San Francisco and Jacobsen, 1985).

An important observation was that the transport system in *A. caulinodans* was repressed by excess succinate (or possibly a product of its metabolism), and was probably controlled independently from succinate dehydrogenase activity. The change in [^{14}C]succinate transport rate from succinate-limited to succinate-excess conditions may have been due to a switch from a high to a low affinity system (Andrews and Lin, 1976; Harder and Dijkhuizen, 1983), or more simply due to a decrease in the number of available/functional transport sites. However, the rate of [^{14}C]succinate transport (regardless of the external succinate concentration) was always sufficient to saturate the incorporation/metabolism rate, suggesting that C_4-dicarboxylate transport was not a limiting factor with respect to growth and so perhaps nitrogen fixation. In contrast sdh activity appeared maximally expressed under conditions of both succinate limitation and excess, and so is perhaps a more likely candidate as the rate limiting step.

CHAPTER 7

General Discussion

Following growth under conditions of succinate limitation *A. caulinodans* exhibited a growth efficiency indicative of an *in situ* P/O quotient of 2.

Whole cells contained cytochromes *b,c,aa*$_3$ and *o*-type, and yielded $\rightarrow H^+/O$ quotients of approximately 6 for the oxidation of endogenous substrates (preliminary results from this study, see also Stam 1986). Comparison of these data with results obtained previously for other species of bacteria suggested the presence of upto three potential sites of respiratory chain-linked energy conservation (Jones *et al.*, 1977). However, the low observed molar growth yields of 42.4 and 27.7 g cell dry wt mol^{-1}, for the Y^{max}succinate and $Y^{max}o_2$ respectively, from succinate-limited cultures when compared to results previously obtained for *Paracoccus denitrificans* (grown under similar conditions) with three sites of proton translocation (Y^{max}succinate and $Y^{max}o_2$ of 58.0 and 36.4 g cell dry wt mol^{-1} respectively) (van Verseveld *et al.*, 1983), indicated an ATP/O quotient of 2 for *A. caulinodans*.

The low molar growth yields may have resulted from a high permeability of the coupling membrane to protons, as suggested by the rapid $\rightarrow H^+/O$ decay ($t_{\frac{1}{2}}$ <15s), and a reflection of either a high $\rightarrow H^+$/ATP quotient, passive leakage through an H^+ uniport system or by electroneutral entry of H^+ with either an anion or through exchange with another cation.

The importance of these results in terms of succinate utilization by bacteroids (assuming the existence of a similar situation *in planta*) is that ATP generation clearly requires a considerable input of oxidizable carbon substrate and is therefore a relatively inefficient process. However, this low apparent efficiency of energy

conservation may be compensated for, firstly, since bacteroids do not grow, much of the ATP generated (allowing for cell maintenance demands *etc*) may be channelled to nitrogenase, and secondly, a calculated ATP/N_2 quotient of 17 for nitrogen-fixing *A. caulinodans* (assuming a P/O quotient of 2) is low compared to other diazotrophs and either suggests an efficient nitrogen-fixing system or perhaps more likely, limitations of the data.

In the presence of excess succinate (oxygen-limited) growth conditions molar growth yields were depressed. The observed decrease was due firstly to the presence of high concentrations of K^+ (derived from KOH addition used to control culture pH), and secondly, through undissociated succinic acid acting as an uncoupling agent.

The concentration of undissociated succinic acid increased when the culture pH decreased according to the Henderson-Hasslebach equation. However, the adverse effect of pH *per se* (increasing H^+ concentration) on molar growth yields was small in comparison to the effects of increasing concentrations of undissociated succinic acid.

The importance of undissociated succinic acid and K^+ concentrations with respect to nitrogen-fixing bacteroids is probably limited. Despite a significant Δ pH between nodule cytosol (pH 5.7) and bacteroid cytoplasm (pH 6.8) the concentration of undissociated succinic acid in the average soybean root nodule is very low (9 μM) (calculated from the data of Streeter, 1987), and so the energy required to expel protons and maintain a bacteroid internal pH of 6.8 is unlikely to effect the overall efficiency of nitrogen fixation under oxygen-sufficient conditions. However, under oxygen-limited conditions the external concentration of succinic acid may increase as a result of succinate metabolism being restricted through the availability of oxygen, and so a higher proportion of ATP would be required to maintain cellular homeostasis than used to supply nitrogenase compared to oxygen-excess conditions.

Interestingly, the uptake of succinate by bacteroids (*via* a proton symport mechanism) would increase the pH of nodule cytosol

and so decrease the concentration of undissociated succinic acid.

The concentration of K^+ that resulted in depression of molar growth yields in free-living *A. caulinodans* was almost certainly in considerable excess to concentrations likely to be encountered by bacteroids, and therefore of limited importance to that system. However, the results highlight the possible dangers that may exist when interpreting molar growth yields of mesophiles grown on succinate or other organic acids that require neutralisation with substantial concentrations of alkali (KOH or NaOH).

Growth of *A. caulinodans* under nitrogen fixing conditions showed that such cultures were sensitive to elevated dissolved oxygen concentrations, as shown by decreases in molar growth yields and growth rate.

DOT values tolerated by *A. caulinodans* were still considerably higher than reported previously for other rhizobia grown under similar conditions i.e. up to 5% DOT (11.5 μM), but were lower than previously reported for this strain (28-40 μM) by Stam *et al.* (1984) and Gebhardt *et al.* (1984). The observed differences between the results reported by the latter authors and those reported in this thesis may have resulted from the different methods used to control the culture DOT.

The increase in respiration rate of *A. caulinodans* in response to increasing DOT was similar in many respects to the process of respiratory protection observed in *Azotobacter* spp. (see Yates and Jones, 1974). However, differences were highlighted with *A. caulinodans* in cytochrome profile, and a higher overall sensitivity to dissolved oxygen concentration.

The significance of increased oxygen tolerance in bacteroids of *A. caulinodans* is unclear (assuming a similar response exists, see Bergersen and Turner, 1975), as both root and stem nodules of *Sesbania rostrata* contain significant amounts of leghaemoglobin, which would maintain a steady-state dissolved oxygen concentration at the bacteroid surface of approximately 7 nM (see Appelby, 1984).

Bacteroid "respiratory protection" may be an important factor when the concentration of leghaemoglobin is low e.g. in young developing nodules, or when the dissolved oxygen level in the nodule cytosol reaches a concentration sufficient to saturate leghaemoglobin e.g. in the vicinity of air tubules.

The maximum rate of succinate utilisation in free-living *A. caulinodans* appeared to be regulated at the level of succinate dehydrogenase (sdh), rather than at the level of transport.

Under both succinate-excess and -limited growth conditions the minimum rate of succinate transport was always sufficient to satisfy the *in situ* qsuccinate. The rate of transport was however greater under succinate limitation than during growth with excess succinate, which suggested a regulatory response of the transport system with respect to succinate (or a product of its metabolism).

The activity of succinate dehydrogenase remained the same during growth under succinate-excess, succinate-limited conditions and with alternative carbon sources. A constant rate of sdh activity would place an upper limit on the rate of succinate metabolism but would not necessarily control it. Fine regulation of succinate metabolism must therefore occur further down the metabolic pathway, perhaps at the level of isocitrate dehydrogenase (Weitzman and Jones, 1968).

The apparent coarse level of control on the entry of succinate into the cell of free-living *A. caulinodans* may be reflected in the accumulation of poly-β-hydroxybutyrate (PHB) under sulphate-limited growth conditions (see also Stam, 1986).

The importance of the findings outlined above with repect to bacteroids of *A. caulinodans* (assuming the existence of a similar system) is that the ability to transport succinate is unlikely to limit the rate of nitrogen fixation unless the external concentration was below the K_m of the transporter. However, the maximum rate of nitrogen fixation may possibly be governed by succinate dehydrogenase activity or the other enzymes involved in dicarboxylate

metabolism.

Coarse control of the initial stages of succinate metabolism in *A. caulinodans* (transport and sdh activity), may suggest that in bacteroids the formation of PHB as a sink for excess reducing power and carbon may not simply reflect oxygen limitation, but may occur during aerobic carbon-excess conditions as well, however, low detectable concentrations of succinate (compared to other potential carbon substrates) present in root nodule cytosol (Streeter, 1987), suggest that under conditions of oxygen-excess the bacteroids are probably carbon-limited.

APPENDIX 1

Steady-state fermentation calculations

1. Gas-flow corrected to STP.

Eq.1

$$\text{STP Gas-flow} = \text{Obs. gas-flow} \times \frac{(\text{atm P} + \text{Man P})}{760 \text{ mm Hg.}} \times \frac{273.15}{\text{Gas T °C}}$$

Units = $1h^{-1}$.

2. Rate of oxygen consumption.

Eq 2.

$$\left(\frac{\text{\% inlet oxygen - \% outlet oxygen}}{100}\right) \times \text{Eq 1.} \times \frac{32}{22.414}$$

Units = g oxygen h^{-1}.

3. Rate of carbon dioxide production.

Eq 3.

$$\left(\frac{\text{\% outlet } CO_2 \text{ - \% inlet } CO_2}{100}\right) \times \text{Eq 1.} \times \frac{44}{22.414}$$

Units = g carbon dioxide h^{-1}.

$$\text{Grammes carbon dioxide carbon } h^{-1} = \text{Eq 3.} \times \frac{12}{44} \quad ; \qquad \text{Eq 4.}$$

4. Rate of succinate consumption.

Eq 5.

{Input succ. (gl^{-1}) x inflow (lh^{-1})} - {Output succ. x outflow}

Units = g succinate h^{-1}.

Grammes succinate carbon h^{-1} = Eq 5. x $\frac{12}{118}$; Eq 6.

5. Rate of supernatant carbon production.

Eq 7.

{Supernatant C. - Output medium C.(gl^{-1})} x Outflow (lh^{-1})}.

Units = $(gl)^{-1}$ h^{-1}.

6. Rate of biomass production.

Eq 8.

$$\frac{\{\text{Broth carbon - supernatant carbon } (gl^{-1})\} \times \text{Outflow } (lh^{-1})}{\frac{\{\% \text{ Carbon in biomass}\}}{100}}$$

Units = g cells h^{-1}.

7. Dilution rate.

Eq 9.

$$\frac{\text{liquid outflow } (lh^{-1})}{\text{Fermenter volume } (l)}.$$

Units = lh^{-1}.

8. Carbon recovery.

$$\frac{\text{Eq 5. + Eq 8. + Eq 9.}}{\text{Eq 7.}} = 1.0$$

9. **Specific rates of substrate consumption/production (q).**

$$q\,O_2 : \frac{\text{Eq 2.} \times \frac{1000}{32}}{\text{Eq 8.}} = \text{mmol } O_2\ h^{-1}\ (\text{g cell dry wt.})^{-1}.$$

$$q\,CO_2 : \frac{\text{Eq 3.} \times \frac{1000}{44}}{\text{Eq 8.}} = \text{mmol } CO_2\ h^{-1}\ (\text{g cell dry wt.})^{-1}.$$

$$q\ \text{Succinate} : \frac{\text{Eq 5.} \times \frac{1000}{118}}{\text{Eq 8.}} = \text{mmol succ } h^{-1}\ (\text{g cell dry wt.})^{-1}$$

10. **Molar growth yields (Y).**

$$Y\ (\text{g cell dry wt. mol}^{-1}). = \frac{\text{Dilution rate } (h^{-1})}{\left\{\frac{(q)}{1000}\right\}}$$

APPENDIX 2

Calculation of theoretical molar growth yields for succinate-limited grown *A. caulinodans*

For the complete dissimilation of succinate in to cell material and carbon dioxide the following equation can be constructed:

$$(a)C_4H_6O_4 + (b)O_2 + (z)NH_4^+ \rightarrow [CH_xO_yN_z] + (d)CO_2 + (e)H_2O \quad \text{eq1.}$$

(where $[CH_xO_yN_z]$ is the molecular formula of the cell derived from elemental analysis, for which the molecular weight is M).

For a given observed Ysuccinate: $A = \frac{M}{Ysucc}$ eq2.
and where A = assimilated succinate

Theoretical carbon balance:

$$(A \times 4) = 1 + d \quad \text{eq3.}$$

Theoretical hydrogen balance:

$$(A \times 6) + (z \times 4) = x + (2 \times e) \quad \text{eq4.}$$

Theoretical oxygen balance:

$$(A \times 4) + (b \times 2) = y + (2 \times d) + (e) \quad \text{eq5.}$$

Solve for **d**, **e** and **b** respectively.

The theoretical yields are given by:

$$\frac{M}{d} = Yco_2 \; ; \; \frac{M}{b} = Yo_2$$

APPENDIX 3

Calculation of undissociated succinic acid concentration

Succinic acid is a weak organic acid with two dissociation constants (K_a1 and K_a2), the concentration of undissociated acid being dependent upon the pH of the solution.

Definitions.

1. $pK_a = -(\log_{10} K_a)$.
2. $[H^+] = 10^{-pH}$ M
3. $C_4H_6O_4 = H_2A$ (undissociated acid).
4. $C_4H_5O_4^- = HA^-$ (conjugate base 1).
5. $C_4H_4O_4^{2-} = A^{2-}$ (conjugate base 2).

Then:

$$H_2A \xrightarrow[pK_a\ 1]{} H^+ + HA^- \xrightarrow[pK_a\ 2]{} H^+ + A^{2-}$$

$pK_a\ 1 = 4.16$

$pK_a\ 2 = 5.61$

The total concentration of succinic acid in solution is the sum of the concentrations of the undissociated acid and the two conjugate bases :

$$[\text{Total}] = [H_2A] + [HA^-] + [A^{2-}] \qquad \text{Equation 1.}$$

$$pK_a\ 1 = \left(\frac{[HA^-] \times [H^+]}{[H_2A]} \right) \qquad \text{Equation 2.}$$

From Equation 2.

$$[H_2A] = \left(\frac{[HA^-] \times [H^+]}{pK_a\ 1} \right) \qquad \text{Equation 3.}$$

$$pK_a\ 2 = \left(\frac{[A^{2-}] \times [H^+]}{[HA^-]} \right) \qquad \text{Equation 4.}$$

From Equation 4.

$$[A^{2-}] = \left(\frac{pK_a\ 2 \times [HA^-]}{[H^+]} \right) \qquad \text{Equation 5.}$$

From Equation 1 & Equation 5.

$$[\text{Total}] = [H_2A] + [HA^-] + \left(\frac{pK_a\ 2 \times [HA^-]}{[H^+]} \right) \qquad \text{Equation 6.}$$

From Equation 6.

$$[H_2A] = [\text{Total}] - [HA^-] - \left(\frac{pK_a\ 2 \times [HA^-]}{[H^+]} \right) \qquad \text{Equation 7.}$$

If [Total], $[H^+]$, pK_a 1 and pK_a 2 are known then equation 3 and equation 4 are simultaneous to solve for $[H_2A]$.

From Equation 2.

$$[HA^-] = \left(\frac{pK_a\ 1 \times [H_2A]}{[H^+]} \right) \qquad \text{Equation 8.}$$

Therefore from Equation 7 & 8.

Equation 9.

$$[H_2A] = [\text{Total}] - \left(\frac{pK_a\ 1 \times [H_2A]}{[H^+]} \right) - \left(\frac{pK_a\ 2 \times pK_a\ 1 \times [H_2A]}{[H^+]^2} \right)$$

From Equation 9.

Equation 10.

$$[\text{Total}] = [H_2A] \times \left[1 + \left(\frac{pK_a\ 1}{[H^+]} \right) \right] + \left(\frac{pK_a\ 2 \times pK_a\ 1}{[H^+]^2} \right)$$

Re-arranging Equation 10.

Equation 11.

$$[H_2A] = \frac{[Total]}{\left[1 + \left(\frac{pK_a\ 1}{[H^+]}\right)\right] + \left(\frac{pK_a\ 2 \times pK_a\ 1}{[H^+]^2}\right)}$$

From Equation 1 & 11.

Equation 12.

$$[H_2A] = \frac{[A^{2-}] + [HA^-] + [H_2A]}{\left[1 + \left(\frac{pK_a\ 1}{[H^+]}\right)\right] + \left(\frac{pK_a\ 2 \times pK_a\ 1}{[H^+]^2}\right)}$$

From Equation 12.

Equation 13.

$$[H_2A] = \frac{[A^{2-}] + [HA^-] + [H_2A]}{\left[1 + \left(\frac{10^{-pKa\ 1}}{10^{-pH}}\right)\right] + \left(\frac{10^{-pKa\ 2} \times 10^{-pKa\ 1}}{[10^{-pH}]^2}\right)}$$

Substitution of the appropriate extracellular total succinate concentration, pK_a and pH values into Equation 13 yields the concentration of undissociated succinic acid in solution.

Therefore, a 1M solution of succinic acid contains :-

at pH 6.8, 139 μM $[H_2A]$

and at pH 5.7, 12.8 mM $[H_2A]$

Calculation of the potassium ion concentration present at various pH values was derived firstly, by solving Equation 13 for the undissociated succinic acid concentration and then substituting the value into the Henderson-Hasselbach equation and solving for the two conjugate base concentrations :

Equation 14.

$$\text{Log}_{10}\frac{[HA^-]}{[H_2A]} = pH - pK_a\ 1$$

Equation 15.

$$\text{Log}_{10}\frac{[A^{2-}]}{[HA^-]} = pH - pK_a\ 2$$

Calculation of the conjugate base concentrations and substitution into Equation 16 yields the K^+ ion concentration :

Equation 16.

$$[HA^-] + 2[A^{2-}] = [K^+]$$

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