Utilization of C_1 compounds by *Frankia* and effects of CO_2 enrichment of the rooting medium on growth and N_2-fixation in *Alnus glutinosa* L. Gaertn.

By

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CONTENTS.

Summary.

In the first part of this project, the effects of CO_2 levels, that may be encountered in the soil atmosphere, on N_2-fixation and growth of the alder plant *Alnus glutinosa* maintained in mineral N-free culture in perlite or water culture, pH 6.3, were examined.

In short-term experiments, enhancing the root system gas phase with CO_2 concentrations within the range of 1.1 to 1.75% CO_2 increased nitrogenase activity (C_2H_2 reduction) of intact nodulated plants. At CO_2 levels around ca. 2.5%, activity was inhibited.

In long-term experiments, the main effects on plant growth were: (a) inhibition of growth of all plant parts following removal of CO_2 from the root system aeration stream; (b) a suggestion of growth stimulation when the aeration stream contained 1.5% CO_2; (c) a suggestion of growth inhibition when the aeration stream contained 5% CO_2; (d) parallel effects on plant total nitrogen content and on whole plant growth, [the specific nitrogen content ($mg.g^{-1}$ plant dwt.) of plant tissues was slightly affected by the treatments]; and (e) a slight increase in the nitrogen fixed per unit weight of nodules, in plants receiving elevated CO_2, especially 1.5% CO_2 when the specific activity was 40% higher than for nodules of control plants.

The results have been interpreted as suggesting that N_2-fixation and growth of alders in some soils may be diminished by moderate levels of CO_2.

The second and third parts of this project were concerned with two main aspects of carbon nutrition in *Frankia*growing *in vitro*.

The ability of *Frankia* to grow chemoautotrophically utilizing H_2 and CO_2 as sole sources of energy and carbon, respectively, was examined. None of the isolates tested were able to grow on carbon-limited media under an atmosphere enriched with 5% H_2 and 5% CO_2 suggesting that *Frankia* is unable to grow chemoautotrophically. This conclusion was reinforced by enzyme assays data, which showed that although the growth of *Frankia* on complex media (the carbon source was propionate) is

stimulated by elevated CO_2, activity of ribulose bis-phosphate carboxylase (RuBPc) enzyme, a key enzyme for assimilation of CO_2 during autotrophic growth, could not be detected.

The possibility that, like some other actinomycetes, *Frankia* might be able to utilize reduced C_1 compounds as sole carbon source for growth was also examined. Methane supported slight growth of 2 from 8 strains tested and most strains grew slowly on methanol and formate. Chromatography of preparations from incubates of cell-free extracts of methanol-grown *Frankia* with ^{14}C-formaldehyde and ribose 5-phosphate showed the incorporation of radioactivity into compounds with chromatographic properties of phosphorylated sugars. Pre-treatment of reaction products with acid phosphatase, followed by chromatography, located radioactivity in compounds with chromatographic properties of free sugars. The results suggest that in *Frankia*, methanol is assimilated *via* the hexulose mono-phosphate (HMP) pathway. The results also raise the possibility that (a) saprophytic growth of *Frankia* in soils could be supported by C_1-compounds, such as methane and methanol, produced by anaerobic fermentation of organic matter and (b) *in vivo Frankia* growth may be supported by methanol, produced by the degradation of cell wall pectin during the infection process.

Abbreviations.

ATP	Adenosine triphosphate.
°C	Degrees centigrade.
CaPs	Carbamyl phosphate synthetase.
d.p.m	Disintegrations per minute
D.W.	Dry weight.
F.W.	Fresh weight.
g	gram(s).
l	Litre.
mg	Milligram.
min.	Minute.
ml	Millilitre.
nmol	Nanomole.
PEP	Phospho*enol*pyruvate.
PEPc	Phospho*enol*pyruvate carboxylase.
p.p.m	Parts per million.
TCA	Tricarboxylic acid cycle.
µCi	Micro-Curie
µg	Microgram.
µm	Micrometre.
µmol	Micromole.
v/v	Volume per volume.
W. m^{-2}	Watt per square metre.

Statistical analyses

Statistical analyses of data were carried out using the statistical package STATGRAF.

The mean and its standard error formulas were:

$$\bar{X} = \frac{\Sigma x}{n} \text{ ; and } \qquad SE = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n(n-1)}}$$

Where: n = number of values of x, Σx = sum of values, $\bar{X}$ = the mean of values, Σx^2 = sum of squares of values, $(\Sigma x)^2$ = square of the sum of values and SE = the standard error of the mean.

When comparing two samples for statistical significance the *t*-test method was used to determine the difference between their means and the value of *t*.

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\frac{(S_1)^2}{n_1} + \frac{(S_2)^2}{n_2}}}$$

Where: $(S_1)^2 = \frac{\Sigma(x_1)^2 - \frac{(\Sigma x_1)^2}{n_1}}{n_1 - 1}$; $(S_2)^2 = \frac{\Sigma(x_2)^2 - \frac{(\Sigma x_2)^2}{n_2}}{n_2 - 1}$.

Where: $\bar{X}_1$ and $\bar{X}_2$ = the means of samples 1 and 2, n_1 and n_2 = the number of values of samples 1 and 2, and $(S_1)^2$ and $(S_2)^2$ = the variances of samples 1 and 2, respectively.

***CHAPTER ONE*:**

GENERAL INTRODUCTION

1.1. Occurrence and history.

Nitrogen is the fourth most abundant elemental constituent of living organisms, being outranked only by carbon, hydrogen and oxygen. In general, plants contain 1 to 5% N on a dry matter basis, with the lower values being common in the vegetative tissues and the upper in seeds as these are usually rich in proteins. Therefore, it is no surprise that in most environments, availability of nitrogen in a suitable form for assimilation is considered to be the factor which most often limits plant growth.

Plants obtain most of their nitrogen from the soil, where nitrate is the most common form of available nitrogen for plant growth. However, nitrates are extremely soluble and thus are readily leached from soils as well as being lost as a result of assimilation into organic matter and by denitrification due to microbial activity. In the absence of available N, plants depend on N from soil reserves but this is not immediately available to plants because it is bound up in soil organic matter. The rate of decomposition of these reserves to produce plant-available N depends upon temperature, pH and the microorganisms present.

In addition to these major sources of N, some plants have adapted to the environments they occupy to supplement their N requirements. For instance, carnivorous plants can utilize nitrogenous compounds from organisms that they can trap. Other plants can obtain extra nitrogen from the atmosphere. However, although dinitrogen gas comprises about 78% of the atmosphere, plants can not benefit from this atmospheric nitrogen unless it is fixed, that is, reduced to ammonia which is then combined with other chemical species, generally organic acids, to be used in the synthesis of proteins. Biological fixation of atmospheric nitrogen (BNF) is accomplished by the reduction of N_2 by the enzyme complex "nitrogenase" to form two molecules of NH_3 and one of H_2 gas - [N_2 + 8 H^+ + 8 e^- + 16 ATP $\xrightarrow{\text{Nitrogenase}}$ 2 NH_3 + H_2 + 16 ADP + 16 Pi]. However, because nitrogenase occurs only in certain prokaryotic organisms (bacteria and cyanobacteria),

eukaryotic organisms like higher plants can not benefit from BNF unless they live in association (symbiosis) with these nitrogenase-possessing bacteria.

Principally, there are two major types of bacterial-higher plant symbiosis: (a), in the case of ectosymbioses the N_2-fixing microsymbiont (cyanobacteria) lives extracellularly and (b), in the case of endosymbioses the N_2-fixing microsymbiont (rhizobia or *Frankia*) is found intracellularly within the cells of nodules, which are formed on the roots of the host plant (angiosperms). Genera of the family Rhizobiaceae nodulate species from the Leguminoseae, e.g. *Glycine*, *Phaseolus,* and *Pisum* sp.; the only exception so far reported to restricted nodulation is the nodulation of *Parasponia rugosa* from the non-leguminous family "Ulmaceae" (Trinick, 1973; Akkermans *et al.*, 1978). Species of the genus *Frankia* nodulate a wide range of non-leguminous plants. While the associations involving cyanobacteria and rhizobia have been studied extensively for decades, the study of the *Frankia*-nonlegume symbiosis was hampered until the late 1970's by the repeated failure of attempts to isolate a microsymbiont that fulfilled Koch's postulates (see Carpenter & Robertson, 1983).

Reports of the presence of nodular structures on the roots of non-leguminous plants began to appear during the 19th century, firstly in *Alnus glutinosa* by Meyen (1829), and by the end of the century there were records of nodulation in many species belonging to the genera *Alnus, Casuarina*, *Ceanothus*, *Elaeagnus*, *Hippophaë* and *Myrica* (Quispel, 1990). Also around that time a series of experiments, in which non-leguminous plants were grown in N-free sandy soil or N-free nutrient solutions, demonstrated that plants bearing nodules grew much better than those without nodules (Dinger, 1895; Mayer, 1895; Hiltner, 1896, 1898). Although the N-content of plant material was not determined, the continuing good growth of these nodulated non-leguminous plants without any addition of nitrogenous salts convinced Nobbe & Hiltner (1904) that, like nodulated legumes, they were fixing atmospheric nitrogen. Efforts to identify the microorganism present in the nodules were unsuccessful, possibly due to the lack of adequate

optical equipment at that time. However, despite this handicap Brunchorst (1886-88) observed, correctly as it was proved later (Becking *et al.*, 1964; Silver, 1964), that the microsymbiont had a hyphal nature.

Little work was done on the *Frankia*-nonlegume association during the first half of this century. Nevertheless, this period was highlighted by some important contributions to the cytology of non-leguminous root nodules. Shibata (1902, 1917) was the first to report that the microsymbiont in the nodules of a number of non-leguminous species resembled actinomycetous organisms. These observations were supported by later studies of Krebber (1932) and Schaede (1933, 1938).

The use of the $^{15}N_2$ technique at the beginning of the second half of this century (Virtanen *et al.*, 1954; Bond *et al.*, 1954) and the acetylene reduction method a few years later (Stewart *et al*, 1967) provided unquestionable proof of N_2 fixation by nodulated non-leguminous plants. Furthermore, the availability of these techniques made it possible for the first time to carry out short-term tests for N_2-fixation on nodules detached from field- or laboratory-grown plants. In this way it was demonstrated that N_2-fixation takes place in nodules rather than root cells. This period was also marked by the first electron microscopic studies on non-leguminous root nodules (Becking *et al.*, 1964; Silver, 1964) which further supported the observations of Brunchorst (1886-88; Shibata, 1902, 1917) and most remarkably detected the vesicle structures which were shown later to be the site of nitrogen fixation (Gardner, 1976; Mian *et al.*, 1976).

As part of the International Biological Program (IBP), which was established in 1964 and continued till 1973, a search for further examples of nodulation among non-leguminous plants was organised. This search, carried out by many researchers around the world, resulted in the addition of new species and even genera to the list of non-leguminous plants nodulated by *Frankia* (Bond, 1976).

The year of 1978, however, is a major milestone in the history of the study of the *Frankia*-nonlegume symbiosis, for two reasons. Firstly, at a meeting in Massachusetts, USA, a new name "Actinorhizal plants" was given to non-

leguminous plants nodulated by *Frankia* to distinguish them from the non-leguminous *Parasponia*, which is nodulated by *Rhizobium* (Tjepkema & Torrey, 1979). The name is formed from the roots "*actino*" for the actinomycete *Frankia* and "*rhiza*" for the host plant root bearing the nodules. Secondly and more importantly, as already mentioned the study of the *Frankia*-nonlegume symbiosis has lagged well behind that of its counterparts largely because of the repeated failure of attempts to isolate and grow the actinomycetous microsymbiont in pure culture. These unsuccessful studies, dating back to as early as 1910 (Peklo), have been thoroughly reviewed (Bond, 1963, 1967; Baker & Torrey, 1979); therefore, a review of all these studies here is quite unnecessary. One particular report worth mentioning, however, is that of Pommer (1959) who reported the isolation of an actinomycete-like organism from root nodules of *Alnus* plants. The isolates were cultivated on a plain glucose-asparagine-agar nutrient medium and then used successfully for re-infecting the host roots with the production of nodules. Although Quispel (1960), who repeated Pommer's experiments, criticised Pommer's report on the basis that the medium used was too simple, he suggested that these claims deserve serious considerations and urgent repitition. Unfortunately, Pommer's isolates were sent to Ettlinger and Zahner in Zurich, who were interested in testing them for antibiotic production, and these isolates were subsequently lost or discarded (Lechevalier & Lechevalier, 1990). Thus, after many unsuccessful atempts to isolate the endophyte, (Becking, 1970) decided that *Frankia* can not exhibit extra-nodular growth and suggested that nodule-forming actinomycetes are obligate symbionts. In 1978, however, Callaham *et al.* (1978) proved that this was not the case by successfully isolating and cultivating a *Frankia* isolate from the sweet fern "*Comptonia peregrina*" root nodules. Ironically, the characteristics (eg. the sporangia produced) of this isolate which formed the basis of the currently accepted definition of a *Frankia* strain and the culture medium used were similar to those described in 1959 by Pommer (Quispel, 1990), making it more regrettable that Pommer's isolates were lost.

Callaham's achievement sparked new interest in the *Frankia*-nonlegume symbiosis research and as a result many new *Frankia* strains have been isolated from root nodules of various actinorhizal plants (see Longeri & Abarzua, 1989). The main methods and media currently used in the isolation and cultivation "*in vitro*" of *Frankia* have been thoroughly reviewed (e.g., Carpenter & Robertson, 1983; Hooker, 1987). The infection pathways have also been reviewed recently (Newcomb & Wood, 1987; Berry & Sunell, 1990). This new interest has also led to the discovery of new actinorhizal plant species. So far 25 genera belonging to 8 families have been shown to include nodulating actinorhizal species and these are listed in Table 1 (see next page).

1.2. Diversity, common habitat and importance of actinorhizal plants.

Despite their taxonomic diversity, occurring in no less than 8 families as opposed to the *Rhizobium* symbiosis in which all genera, but one, belong to the Leguminoseae family, the actinorhizal plants have many common features. They are all perennial dicotyledons that may survive for many years (Akkermans & van Dijk, 1981; Bond, 1983) and all but *Datisca*, which has herbaceous shoots (Hafeez *et al.*, 1984), are woody shrubs or trees (Bond, 1983).

In the last decade several reviews (Akkermans & van Dijk, 1981; Bond, 1983; Tjepkema & Schwintzer, 1986; Diem & Dommergues, 1990; Hibbs & Cromack, 1990; Wheeler & Miller, 1990) have provided comprehensive information on the evolution, distribution and practical uses of actinorhizal plants.

In terms of evolution, in the glacial and post-glacial times, actinorhizal plants were abundant and widely distributed in Europe and North America, where they colonized nitrogen-poor deposits and accelerated soil development by adding nitrogen, but were later displaced as increasing soil nitrogen permitted non-nitrogen fixing plants to flourish (Silvester, 1977). In modern times, although they are found on every continent except Antarctica, they are much less abundant, being found in

Table 1. Currently identified actinorhizal plants nodulated by the nitrogen-fixing actinomycete *Frankia.*[1]

Order	Family	Genus	No. of species
Fagales	Betulaceae	*Alnus*	47
Casuarinales	Casuarinaceae	*Allocasuarina*	54
		Casuarina	16
		Ceuthostoma	02
		Gymnostoma	18
Ranunculales	Coriariaceae	*Coriaria*	16
Violales	Datiscaceae	*Datisca*	02
Protales	Elaeagnaceae	*Elaeagnus*	38
		Hippophaë	02
		Shepherdia	02
Myricales	Myricaceae	*Comptonia*	01
		Myrica	28
Rhamnales	Rhamnaceae	*Adolphia*[2]	01
		Ceanothus	31
		Colletia	04
		Discaria	05
		Kenthrotamnus	01
		Retanilla	02
		Talguanea	01
		Trevoa	02
Rosales	Rosaceae	*Cercocarpus*	04
		Chamaebatia	01
		Cowania	01
		Dryas	03
		Purshia	02
		Rubus[3]	02

[1]. Complied from Baker & Schwintzer (1990) and Bousquet & Lalonde (1990). [2]. Reported recently by Cruz-Crisneros & Valdes (1991). [3]. The genus *Rubus* is included in the table on the basis of earlier reports of nodulation in this genus (Bond, 1976; Becking, 1984). However, the validity of these reports has been seriously questioned by Stowers (1985_a) who found that *Myrica rubra* is the only actinorhizal plant in the Indonesian site from which *Rubus ellipticus* nodules were reported to be collected by Becking (1984).

rather harsh environments such as sandy and gravelly sites, along streams and in wetlands, and other nitrogen-poor sites (Silvester, 1977). Studies have shown that human activities have also helped to change actinorhizal plants distribution over the years, introducing new genera into different regions. The geographical ranges of *Casuarina* and *Elaeagnus*, originally from Australia and Eurasia, respectively, have

in particular been greatly expanded through these activities. More genera (3 in total) have been introduced into Africa, which had only one native genus "*Myrica*", than into any other continent (Baker & Schwintzer, 1990).

Ecologically, the current distribution of actinorhizal plants highlight their great ability to adapt to different and severe environments (Bond, 1983). For example, with respect to temperature, species such as *Alnus incana*, *Myrica gale*, and *Hippophaë rhamnoides* are all found in the Arctic Circle, where temperatures are very low. At the other extreme, Casuarinas have to tolerate temperatures of 35 °C or more in Australia and Indonesia, where they are abundant. With respect to adaptation to badly drained soils and drought, on the one hand, inspection of *Myrica gale* in France, Scandinavia and Britain showed that nodulation was best in wet soils. *Alnus glutinosa* and some other species of *Alnus* also prefer wet soils and are abundant on the fringes of Scottish lochs. On the other hand, species of *Casuarina* and *Colletia* grow in areas of low rainfall in Australia and South America, respectively, and species of *Ceanothus* and *Purshia* survive in near desert conditions in the USA.

With the exception of some species such as *Hippophaë rhamnoides* which is used for its fruits in Eastern Europe and China (Wheeler & Miller, 1990), actinorhizal plants are not important sources of food for human. Nevertheless, they are of considerable economic importance, particularly in forestry, in biomass production, and in reclamation and amenity planting. In forestry, *Alnus rubra* is a major source for timber and pulpwood in the Pacific Northwest forests (Hibbs & Cromack, 1990) and *Casuarina*, which is widely distributed in tropical regions, is reported to be the world's best source for timber and fuel-wood from actinorhizal plants (National Academy of Sciences, 1980; Gauthier *et al.*, 1985). In biomass production, many actinorhizal plants can be interplanted with crop trees or used in rotation with them to improve production on nitrogen-poor sites (Gordon *et al.*, 1979). For instance, *Alnus rubra* is used as a nurse crop for the lumber species "Douglas fir", which uses the fixed nitrogen released by decaying alder leaf litter

(Atkinson *et al.*, 1979) and *Myrica gale* returns about 70%, annually, of the N it fixes to the soil and thus is an important source for combined nitrogen (Schwintzer, 1984). With respect to land reclamation and amenity planting, *Alnus*, *Elaeagnus*, and *Hippophaë* have been widely used for land stabilization in Western Europe (Wheeler & Miller, 1990) and *Casuarina* can be used as a wind break or to controll erosion (National Academy of Sciences, 1980). Furthermore, *Comptonia peregrina*, and *Elaeagnus umbellata* are used for re-vegetation and landscaping of nitrogen-poor sites such as strip mines and highway roadsides (Carpenter & Hensley, 1979).

1.3. Characteristics of *Frankia*.

Studies, carried out in the last decade or so, have shown that *Frankia* is a heterotrophic, aerobic and some times microaerophilic microorganism. When grown in pure culture, depending on the medium composition, *Frankia* differentiates into three different cell types (vegetative, sporangiae and vesicular). With some exceptions, these cells can also be observed in the symbiotic state. When grown in medium containing combined nitrogen compounds, *Frankia* grows as a filamentous mat consisting of branched, septate hyphae with sporangia developing terminally and at intercalary positions (Newcomb *et al.*, 1979; Horrière *et al.*, 1983). The sporangia vary both in size and shape but are often club-shaped and measure ~10 µm x 30-50 µm (Newcomb *et al.*, 1979). However, if combined nitrogen is omitted from the growth medium, spherical structures are borne on short lateral branches of the vegetative mycelium. These spherical structures, about 3-5 µm in diameter, are termed *Frankia* vesicles and are capable of reducing acetylene, i.e., fixing nitrogen (Tjepkema *et al.*, 1980). In the symbiotic state the shape of vesicles is determined by the host plant. This statement is supported by the observation that vesicles produced by a strain, isolated from *Comptonia peregrina*, are spherical in *Alnus glutinosa* but club-shaped in its original host *Comptonia peregrina* (Lalonde, 1979).

When observed either in culture or within nodules, the vesicles are

surrounded by a laminated thick layer (Torrey & Callaham, 1982), the vesicle envelope, which has been suggested to act as a protector of nitrogenase from inactivation by oxygen (Tjepkema *et al.*, 1980). This suggestion is supported by recent data showing that the number of laminations increases with increasing oxygen levels (Parsons *et al.*, 1987). While N_2-fixing *Frankia* always produces vesicles in culture, this is not always the case for the organism in the symbiotic state. Thus, in the *Frankia-Casuarina* symbiosis, where no vesicle and hence no envelope is produced, Berg (1983) suggested that protection from oxygen may be achieved *via* the specially modified cell walls of the infected cortical cells of *Casuarina.* These cell walls were lignified or suberised or both. Furthermore, *Casuarina* nodules are known to contain haemoglobin at concentrations similar to those of legume nodules, although this O_2 binding pigment is also present in varying quantities in the nodules of other actinorhizal species (Silvester *et al.*, 1990).

Other cultural characteristics such as carbon and nitrogen growth requirements of *Frankia* will be discussed later in chapter 3.

1.4. CO_2 fixation by N_2-fixers.

1.4.1 CO_2 in the atmosphere and soil.

There is considerable evidence that the earth's atmospheric CO_2 content has been increasing, at least for the last 130 years. For example, recent estimates of the global carbon reservoirs have shown that prior to the industrial revolution, about 130 years ago, the air contained about 260 ppm CO_2. More recently, however, it contained 350-360 ppm (Conway *et al.*, 1988; Paul & Clark, 1989) and the $[CO_2]$ is still increasing at the rate of about 1.2 ppm per year (Conway *et al.*, 1988). It has been suggested that if the current use of fossil fuel and the combustive reduction of the world's forest is maintained, the current atmospheric $[CO_2]$ will double by the middle of next century (see Reuveni & Gale, 1985). In addition to the effects of

other environmental factors, this increase could have major effects on growth and production of plants since CO_2 is the carbon substrate of photosynthesis and it also inhibits photorespiration. Indeed, an analysis of more than 430 observations, collected from 70 previous reports of the yield of 37 species grown with CO_2 enrichment, showed that for a 300 ppm augmentation of atmospheric [CO_2] the yields (economic yield and dry weight) of most crops and woody species would probably increase by 33% (Kimball, 1983).

With respect to soil, the CO_2 content of the gaseous phase of soil is affected by many different physical and biological soil factors such as aeration and water content. However, given the continual biological interchange between atmospheric CO_2, land plants and soil that occurs during global carbon cycling one could speculate that changes in atmospheric [CO_2] would also lead to changes in soil carbon reservoirs.

1.4.2. CO_2 fixation by N_2-fixers growing *in vivo* (*in planta*).

Although elevated levels of atmospheric [CO_2] are not likely to have direct effects on biological nitrogen fixation, considerable amounts of data showing that nitrogen fixation is linked to photosynthesis have been published (e.g., Wilson *et al.*, 1933; Phillips *et al.*, 1976; Layzell *et al.*, 1979; Minchin *et al.*, 1981; Ta *et al.*, 1987). Estimates with N_2-fixing legumes suggest that 30% of the carbon gained through photosynthesis in the shoot is used for nodule function and maintenance (Layzell *et al.*, 1979; Minchin *et al.*, 1981). The role of photosynthates supplied to the nodules has been summarized by Rawsthorne et al. (1980) as follows: (a) generation of reducing power and ATP for the nitrogenase system, (b) maintenance of normal host-cell cytosol metabolism, and (c) supply of carbon skeletons, ATP and reducing power for the synthesis of nitrogenous compounds which are then exported back to the growing parts of the plant *via* the xylem.

Carbon is made available for plant growth, not only through photosynthesis but also through a variety of dark CO_2-fixation reactions. This was first

demonstrated about 50 years ago by Ruben & Kamen (1940) who showed non-photosynthetic (dark) fixation of $^{11}CO_2$ by barley root tissues, and by Overstreet *et al.* (1940) who studied the uptake of radioactive bicarbonate by barley plants. Subsequently, the pathways of fixation and their relevance to the physiology of growth of many plant species have been investigated extensively. The occurrence of this process in N_2-fixing plants, specifically, and its relevance to nodule function and plant growth has also been the subject of many investigations, mainly with legumes, and will be discussed in detail in chapter 2.

1.4.3. CO_2 fixation by N_2-fixers growing *in vitro* (in culture).

It has been known for decades that bacteria require CO_2 when growing in culture (Valley & Rettger, 1927). A CO_2 requirement for growth of five different species of rhizobia isolated from root nodules was first demonstrated by Lowe & Evans (1962). The subsequent experiments that led to the discovery that some rhizobia species can grow autotrophically in culture, i.e. utilizing CO_2 as their major carbon source, will be reviewed in detail in chapter 3.

With respect to *Frankia*, enhancement of growth of a strain growing in culture with added CO_2 has been demonstrated (Akkermans *et al.*, 1983) and there is one report which claims that *Frankia* is able to grow under chemoautotrophic conditions; that is utilizing H_2 and CO_2 as energy and carbon sources, respectively (Shipton & Burggraaf, 1983).

1.5. Occurrence of C_1 compounds used for microbial growth.

C_1-compounds are defined by Large (1983) as organic compounds that are more reduced than CO_2 and contain no carbon-carbon bonds, although some contain more than one carbon atom as shown in Table 2.

Table 2. Examples of C_1 compounds and their occurrence.[1]

Compound	Source
Methane, CH_4	Methanogenic bacteria.
Methanol, CH_3OH	Oxidation of atmospheric methane. Methoxy groups of lignin and pectin.
Formate, HCOOH	Industry, microbial action.
Carbon monoxide, CO	Porphyrin decomposition. Vehicle exhausts. Blast furnaces.
Formamide, $HCONH_2$	Synthesis.
Hydrogen cyanide, HCN	Metal extraction. Cyanogenic glycosides.
Monomethylamine, CH_3NH_2	Tanning effluent.
Dimethylamine, $(CH_3)_2NH$	Decaying fish.
Trimethylamine, $(CH_3)_3N$	Decaying fish.
Tetramethylammonium, $(CH_3)_4N^+$	Industrial chemical waste.

[1], Selected from Large & Bamforth (1988).

Methane is the most abundant C_1 compound in nature. In addition to the high quantities present in coal and oil deposits, it is evolved on a large scale as an end product of many fermentations, e.g. during sewage production or ruminant digestion (Quayle, 1972). It is also ubiquitous in anaerobic lakes, ponds and paddy fields, as these are favourite habitats of methanogenic bacteria, the main source of

methane (Large & Bamforth, 1988).

Methanol arises in the troposphere (upper atmosphere) from the photo-oxidation of methane then descends in small quantities in rain (Ehhalt, 1976). It is also formed as a result of the activities of certain bacteria on lignin, hemicelluloses and pectin which are important components of the plant biomass (Donnelly *et al.*, 1981; Large & Bamforth, 1988).

Formaldehyde is too reactive to occur at significant concentrations in nature, but formic acid occurs in plant and animal tissues and is frequently formed from various industrial processes or as an end product of carbohydrate fermentation (Quayle, 1972).

Methylated amines or amine oxides occur widely where fish and invertebrates die and where industrial wastes are disposed of.

Other C_1 compounds such as cyanide and carbon monoxide, also are becoming increasingly recognized as occurring on a significant scale in certain environments, particularly in heavy polluted areas (Knowles, 1976; Meyer, 1985).

Utilization of these C_1 compounds, as sole carbon source, by many N_2-fixing bacteria, including actinomycetes to which *Frankia* belong, will be reviewed in chapter 4.

1.6. Outline of the aims of the project.

1.6.1. Experiments described in Chapter 2:

Changes in the CO_2 levels of the rooting medium have been shown to have major effects on N_2-fixation and growth of leguminous plants (Mulder & Van Veen, 1960) (see Introduction to chapter 2). Changes in the CO_2 content of the rooting medium in which alders are grown might be expected to have even greater effects than in legumes, because of (a) the special requirements for CO_2 for the synthesis of citrulline which is the main amino acid of species such as *Alnus glutinosa* (see chapter 2) and (b) the conditions of poor soil aeration in which they frequently grow. In flooded soils, CO_2 levels can rise to 12% and the extracellular levels of CO_2 in flooded tissues can exceed 5 % (Bown, 1985). However, no studies on the effects of changes in the rooting medium CO_2 levels have been reported yet for alders. It is this aspect which has been studied here.

Both short-term effects of changes of CO_2 levels in the rooting medium on nitrogenase activity (C_2H_2 reduction) of *Alnus glutinosa* and long-term effects of different levels of CO_2 in the aeration supply to the root system of water-culture-grown *A. glutinosa* on nitrogen fixation and plant growth are reported.

1.6.2. Experiments described in Chapter 3:

As already mentioned CO_2 requirement for *Frankia* growth and growth of *Frankia* under chemoautotrophic growth have been reported by Akkermans *et al.* (1983) and Shipton Burggraaf (1983), respectively. However, these studies, which will be reviewed in chapter 3, were limited to a small number of strains and have not been repeated or further investigated in detail. Thus a larger number of *Frankia* strains have been tested here, in more detail, for the ability to grow autotrophically.

1.6.3. Experiments described in Chapter 4:

In contrast to early suggestions that *Frankia* could not exhibit extra-nodular growth (Becking, 1970), it is now almost certain that like the majority of actinomycetes *Frankia* also is able to grow saprophytically in soil (see introduction to chapter 4). Furthermore, we have already seen that some of the habitats where C_1 compounds such as methane are ubiquitous (i.e., wet and badly aerated soils) are favourable habitats for growth and nodulation of alders. The ability of other actinomycetes to degrade some plant biomass components, major sources of methanol and some other C_1 compounds, has also been confirmed recently (introduction to chapter 4). In addition, *Frankia* may be able to utilise for growth "*in vivo*" methanol produced by the degradation of cell wall pectin during the infection process since Schouten & Lalonde (cited by Lalonde, 1977) found that the *Alnus glutinosa* root nodules are the site of a high pectolytic activity. These pectolytic enzymes were extractable from the root nodule and were shown to be able to degrade the pectic capsule surrounding the *Alnus glutinosa* endophyte. Pectolytic activity was not detectable in the non-nodulated *Alnus* roots or root apex.

These facts together suggest that like some other actinomycetes *Frankia* also may be able to utilize C_1 compounds such as methane, methanol and formate as sole carbon source for growth. This possibility has been examined here.

CHAPTER TWO:

EFFECTS OF CO_2 ENRICHMENT OF THE ROOTING MEDIUM ON GROWTH AND N_2-FIXATION IN *ALNUS GLUTINOSA*.

2.1. INTRODUCTION.

2.1.1. Effects of CO_2 on growth of non-nodulated plants.

Experiments carried out during the 1940's, utilizing radiolabelled CO_2, showed conclusively that plant roots are capable of fixing CO_2. However, prior to this date, considerable evidence had been obtained from plant growth studies showing that root development was influenced by levels of CO_2 in the rooting medium. The main findings are summarised in Table 3 (pages: 19-21). In the early experiments there was wide variability both in the range of species studied, most of which were non-legumes especially prior to 1955, and in the experimental conditions used. In general, increasing CO_2 levels supplied to the rooting medium, by up to 5% of the aeration gas, resulted in some stimulation of growth (increase in root length or in dry matter). However, there were exceptions, for example; inhibition of maize root dry matter by 0.3% (Knight, 1924); of potato tubers dry matter by 0.8% (Ruck & Dolas, 1954); and of pea root elongation by 1.5% (Stolwijk & Thimann, 1957). By contrast, Arteca *et al.* (1979) found that CO_2 concentrations as high as 45%, in the rooting medium (pH 5.5) stimulated dry matter production and tuberization in potatoes, which are resistant to CO_2 damage.

While differences in the response of different plant species are expected to play a part in the wide variability in the results obtained, variations in many of the experimental conditions used by different researchers also must have considerable effects on these results. For example, experiments have been performed for different durations and this must influence the results obtained. Thus, Mer (1952) found that 2.5%, 5%, and 10% CO_2 concentrations in the aeration stream inhibited the elongation of mesocotyls of oat for the first 3 days; but by day seven, both 2.5% and 5% CO_2 concentrations showed stimulatory effects and at the end of the experiment (day 9) even 10% CO_2 stimulated elongation. Furthermore, some researchers grew

their plants in soil while others used water-culture as nutrient medium. The use of a complex growing medium such as the soil makes it difficult to monitor changes in other factors, such as pH, oxygen concentrations and water movement that could contribute to or reduce the growth effects of changes in the CO_2 concentrations. To minimize these difficulties, some investigators have replaced soil-culture with water-culture methods, although it is obvious that the response of the plant to changes in CO_2 under water-culture conditions is not necessarily the same as it would be when grown in soil.

Physiological experiments to determine the effects of CO_2 concentration in the rooting medium on root growth of legumes were first carried out by Hammond *et al.* (1955), who found that up to 20% CO_2 in the soil air (pH not stated), in which the plants were grown, had little effect on dry matter production of non-nodulated soybeans. Two years later, Stolwijk & Thimann (1957), working with non-nodulated seedlings, grown in nutrient solution (pH 5.9) and supplied with NO_3^- as the nitrogen source, found that the root elongation of several species (*Pisum sativum*, *Vicia faba* and *Phaseolus vulgaris*) was inhibited by 6.5% CO_2. The roots of *Avena sativa* and *Hordeum vulgare* were unaffected by such treatment. In a more detailed analysis of the growth response of pea (*Pisum sativum*), they found that root elongation was stimulated by 0.5% CO_2, but inhibited at levels as low as 1.5%. Somewhat different results were reported for peas grown in a mixture of sand and compost (pH not stated) by Geisler (1967) who noted stimulation in root length and dry matter when CO_2 concentrations were increased up to 2% in the presence of low O_2 concentrations (0 to 7%), but inhibition with 8% CO_2.

In the last 25 years, several investigators have used excised root tissues to study the effects of elevated CO_2 on root growth. Such experiments avoid the possibility that some of the observed effects on root growth could be due to CO_2 transported from the shoots to the roots, although the relevance of the results obtained from the growth of excised roots to growth of whole plants must be

questioned. Splittstoesser (1966) reported a 50% increase in dry weight of excised tomato roots in air compared with roots aerated with CO_2-free air. Furthermore, aeration with air containing 5% CO_2 was found to increase the main root axis length, number and length of lateral roots and fresh weight of excised wheat roots cultured in water culture (pH 4.6 to 4.8), compared with roots grown without aeration, which in turn grew better than roots aerated with CO_2-free air (Talbot & Street, 1968).

In summary, the results of these physiological experiments show that although increasing the CO_2 content of the rooting medium, within certain limits, can stimulate plant growth, the range of CO_2 concentrations that stimulate growth varies with different species and between different experiments.

Table 3. Effects of the rooting medium CO_2 levels on growth of non-nodulated plants.

Plant	Growth medium and its pH	Treatment duration	Stimulatory CO_2 concentrations	Lowest inhibitory CO_2 concentration	Plant parameter measured	Reference
Maize	Soil, pH not stated	7-8 wks	< 0.3%	0.3%	Length of main and lateral root, fresh weight of excised roots and number of lateral roots.	Knight, 1924
Tomato; rice	Water	6 wks	ND	pure CO_2	Plants were killed.	Vlamis &
Barley	"	6 wks	ND	20% (80% O_2)	"	Davis, 1944
Cotton	Water, 5.4 to 5.6	12-14 days	0-15% (not sig.)	30%	Elongation of roots.	Leonard & Pinckard, 1946
Tomato	Water, 4.5	24 hrs	0.1-5%	9%	Root length.	Erickson, 1946
Oat (*Avena*	Not stated	03 days	ND	2.5%	Elongation of mesocotyl.	Mer, 1952
sativa)		07 days	2.5 to 5%	10%	"	
		09 days	2.5 to 10%	ND	"	
		"	2.5% (not sig.)	5%	Elongation of coleoptile.	
Potato	Soil, 7.4 to 8.0	10 days	0.8 to 1.5%**	ND	Number of tubers.	Ruck &
		"	" (not sig.)	ND	Dry weight of roots.	Dolas, 1954.
		"	" (not sig.)	0.8%	Dry weight of tubers and whole plant.	

Continued on next page.....

Table 3. Continued.

Corn; soybean	Soil, pH not stated	03 days	2-20% (not sig).	45% (18% O_2)	Dry weight of plants.	Hammond
Corn	Soil	04 wks	0 to 0.25%	30%	"	*et al.*, 1955
Corn	Water	03 wks	0 to 15%	20%	"	
Oat	Not stated	03 days	ND	5%	Elongation of mesocotyls and coleoptiles.	Mer, 1957
		08 days	5%	ND	Length, Fw. and Dw. of mesocotyls.	
		"	ND	5%	Length, Fw. and Dw. of coleoptiles.	
		3-8 days		5%	Length, Fw. and Dw. of whole plant.	
Wheat	"	3-8 days		5%	Elongation of coleoptiles.	
P. sativum	Water, 5.9	10-15days	0.2 to 0.5%	1.5%	Root elongation	Stolwijk &
P. vulgaris	"	"	ND	6.5%	"	Thimann, 1957.
Peas	Water, 5.3 to 5.9	03 wks	10%*	12.5%	Dry weight of main root.	Geisler, 1963
		"	7.5%*	"	Dry weight of lateral roots.	
		"	10%*	"	Root elongation.	
		"	5%*	"	Number of lateral roots.	
Oat	Distilled water	50-60 hrs	0.5% to 10%	15%	Length of coleoptiles.	Harrison, 1965

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Table 3. Continued.

Tomato	Water	05 days	air*	ND	Dry weight and length of excised roots.	Splittoesser, 1966
Barley; peas	Sand + compost	18 days	2%(0-7% O_2)*	8%	Root length and biomass production.	Geisler, 1967
Wheat	Water, 4.6 to 4.8	14 days	5%*	ND	Length of main and lateral root, fresh weight of excised roots and No. of lateral roots.	Talbot & Street, 1968
Oat	Buffer, 7.5	02 hrs	0.03%*	ND	Elongation of coleoptile.	Bowen *et al.*, 1974.
Potato	Water, 5.5	12 hrs	45%	ND	Dry weight of tubers, roots and shoots and number of tubers.	Arteca, 1979

Abbreviations : wks = weeks, hrs = hours, ND= not determined, and not sig. = not statistically significant ($P<0.05$).

Symbols : (") the same as above; (*) the control was CO_2-free air; (**), the control was non-aerated soil (0.4 to 0.5% CO_2). In the remaining experiments the control was always air.

2.1.2. The causation of CO_2-related growth effects.

2.1.2.1. Effects of dark CO_2 fixation on plant metabolism.

It was presumed from the early experiments, cited before, that the major stimulatory effect of increased levels of CO_2 in the rooting medium is to increase the activity of the CO_2-fixing root carboxylation reactions, thereby providing additional carbon for the synthesis of organic acids that may be respired or used to provide C skeletons for assimilation of ammonia. However, CO_2 has also been suggested to affect plant growth in ways which are not apparently linked directly to carbon metabolism. Investigation by Rayle & Cleland (1970) of the growth response (elongation) of *Avena* coleoptile sections to CO_2-saturated solutions showed a stimulatory effect at acid pH 3.0 that occurred within 1 min of treatment, was insensitive to the protein synthesis inhibitor cycloheximide, and was greater than that obtained with auxins or acid pH alone. These characteristics indicated that the growth response was not dependent on protein synthesis and was not mediated by a metabolic process. The response did not occur at pH values closer to neutral. By contrast, Bown *et al.* (1974) showed that 0.03% CO_2 at pH 7.0 stimulated growth (compared to CO_2-free air) after a lag period of 10 to 15 min. and that the response was inhibited by cycloheximide. This sensitivity to cycloheximide and the large lag period before any measurable effect on growth of supplying 0.03% CO_2 at pH near neutral is in keeping with an effect on metabolism (CO_2 fixation) which in turn results in growth stimulation.

It seems, therefore, that CO_2 can stimulate coleoptile growth by two different mechanisms: (1) an acid pH-dependent stimulation of growth by CO_2-saturated solutions; and (2) a growth stimulation by CO_2 that is not dependent on low pH values, and that involves dark CO_2-fixation, a time-consuming metabolic process. It is unlikely that the stimulation of coleoptile growth at highly acid pH by CO_2-saturated water is of relevance to growth of plant roots under more usual

conditions, so that it is the metabolism of additional fixed CO_2 that usually produces growth stimulation. Bown *et al.* (1974) found further support for a metabolic role of fixed CO_2 in growth stimulation from a second experiment in which malate, the first detectable labelled product on the addition of $H^{14}CO_3$ to *Avena sativa* coleoptile tissue (Bown & Lampman, 1971), was shown to have similar stimulatory effects on growth as that of CO_2.

These observations on the fate of fixed CO_2 are in agreement with earlier reports that showed that fixed CO_2 could be used in the synthesis of tricarboxylic acid cycle (TCA) acids and of amino acids (Poel, 1953; Jackson & Coleman, 1959), and that metabolism of TCA cycle acids could stimulate protein synthesis (Splittstoesser, 1966; Bown & Aung, 1974).

2.1.2.2. Uptake of HCO_3^- and its role in inducing plant-chlorosis and other growth effects.

It is notable, as mentioned before, that most investigators have found that root growth is inhibited when CO_2 concentration in the rooting medium reaches a particular level. It is also notable from Table 3 that different investigators have employed in their experiments rooting media of widely different pH values and in some cases have not reported the pH used at all. Changes in rooting medium pH can have critical effects on a wide range of processes in roots, such as anion and cation absorption, respiration and protein synthesis (Steward & Preston, 1941). In relation to CO_2, pH affects the balance between the various ionic forms of carbonic acid derived from CO_2 equilibration with water and dissociation of the resulting H_2CO_3 ($CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow HCO_3^- + H^+$) within the solution. The relationship between the concentrations of undissolved CO_2 and H_2CO_3, the ions ($HCO_3^- + H^+$), and the pH is given by the Henderson-Hasselbach equation:

$$pH = pK_a + Log_{10} \frac{[HCO_3^-]}{[CO_2 + H_2CO_3]}, \qquad pK = 6.35.$$

The equation shows that as the pH of the solution is raised to the pK_a value (6.35) and beyond, increasing concentrations of HCO_3^- are required to keep the equation in balance.

The uptake of HCO_3^- into roots was well established more than 30 years ago (Poel, 1953; Graf & Arnoff, 1955; Jackson & Coleman, 1959; Bedri *et al.* 1960). A detailed study by Bedri *et al.* (1960) showed that in all the plants tested (bush beans; soybean; avocado; trifoliate orange; and barley) most of the ^{14}C, supplied as $KH^{14}CO_3$ to the nutrient solution with 1 mmol/L KNO_3 as nitrogen source, was detected 12 hours later in the roots (60 to 90%) with considerably less activity detected in the stems and leaves. These early findings were confirmed in recent studies by Wallace *et al.* (1979), who showed a decreasing gradient in ^{11}C from roots to shoots to leaves 2 hours after immersion of the roots of bush beans in a nutrient solution (pH 6.5) containing $KH^{11}CO_3$ or $NaH^{11}CO_3$. When $(NH_4)_2SO_4$ was supplied, ^{11}C movement into the upper part of the plant was enhanced while KNO_3 seemed to decrease the movement. Furthermore, Vapaavuori & Pelkonen (1985) detected ^{14}C label in the leaves and shoots of willow cuttings 6 and 48 hours after feeding $NaH^{14}CO_3$ to the roots in a nutrient solution of pH 5.5. In a second experiment, they showed that the dry matter production of shoots, leaves, roots and the whole plant increased as $NaHCO_3$ concentration was increased up to 0.737 mol m^{-3} (31.1% increase in dry weight of the whole plant compared to controls, which did not receive additional bicarbonate). At higher concentration of $NaHCO_3$ (1.47 mol m-3), dry matter production, especially of shoots and leaves, was less than that at 0.737 mol m-3, but was still almost 26.8 % higher than in the control plants. These results suggest that dry matter production of willow cuttings would be reduced progressively with further increase in $NaHCO_3$ supplied to the roots.

High concentrations of bicarbonate ions have been shown to inhibit grass

and plant growth in numerous studies (Gaugh & Wadeleigh, 1951, Wadleigh & Brown, 1952; Hassen & Overstreet, 1952; Brown & Wadleigh, 1955; Porter & Thorne, 1955; Abdessalam *et al.*, 1965; Woolhouse, 1966; Lee & Woolhouse, 1969_a; Kolesch et al., 1984), causing in many cases symptoms identical to those of plant-chlorosis. Interest in the bicarbonate-induced plant-chlorosis arose from the early observations of Harley & Linder (1945) on the response of apple and pear trees to irrigation waters which varied in their bicarbonate content. They reported that chlorosis was always associated with soils containing higher concentrations of bicarbonate. Porter & Thorne (1955) found that when bean and tomato plants were grown in culture solutions of constant pH (7.8), increased concentrations of $NaHCO_3$, resulting from changes in the CO_2 content of the aeration gas, decreased the dry matter production (leaves, stems and roots) and increased both chlorosis and iron concentrations in leaf and stem tissues. Varying the pH (7.3-8.0) with various CO_2 concentrations at constant $NaHCO_3$ content produced no significant effects upon the plants, indicating, as suggested by many earlier workers (e.g., Gaugh & Wadeleigh, 1951; Brown & Wadleigh, 1955), that it is HCO_3^- rather than high pH that induces chlorosis.

High concentrations of bicarbonate have also been shown to have major effects on the solubility of minerals in the nutrient solution, especially phosphorus, and on their movement into the individual parts of the plant (Steward & Preston, 1941; Greenwald, 1945; Biddulph & Woodbridge, 1952; Goss & Romney, 1959; Wallihan, 1959; Miller *et al.*, 1960). For example; Miller *et al.* (1960) reported that addition of HCO_3^- to a nutrient solution resulted in a 10-fold increase in P solubility, compared with a solution that did not receive additional HCO_3. These observations led to the suggestion that chlorosis may follow increased solubility and uptake of P in the presence of high concentrations of HCO_3^- (DeKock, 1955; Brown *et al.*, 1959; Brown, 1960). However, Coulombe *et al.* (1984) showed that this is unlikely to be the case since addition of HCO_3^- to nutrient solutions was necessary

to induce chlorosis in *Wayne*, a soybean chlorosis susceptible cultivar, both at low and high P content in the nutrient solution. Their foliar analysis also showed that P in leaves was decreased by HCO_3^- at 400 µmol P and was unchanged at 10 µmol P, indicating that chlorosis in *Wayne* in the presence of HCO_3^- is not the result of increased solubility and enhanced plant uptake of P. This is in agreement with the results of Mengel *et al.* (1984) who showed that bicarbonate was the major factor inducing iron chlorosis in vine grapes on calcareous soil. In fact, they concluded from their data that the high P content frequently found in chlorotic leaves could be the result and not the cause of Fe chlorosis. However, the role of phosphorus in the plant-chlorosis phenomenon is not completely clear since it may for example, impair iron nutrition by precipitation as Fe-phosphates in the growth medium and at the root surface or immobilise iron within the plant (Biddulph & Woodbridge, 1952; Cumbus *at a*l., 1977).

Many researchers have related plant-chlorosis directly or indirectly to iron deficiency. However, some data are in conflict with this conclusion since they have shown that the leaves of plants suffering from chlorosis have a total iron content similar to or even higher than that of green plants (Bennett, 1945; Porter & Thorne, 1955; Coulombe *et al.*, 1984; Kolesch *et a*l., 1984), indicating that chlorosis could result, at least in some cases, from physiological inactivation of iron in the plant tissues rather than failure of its absorption. Chelation of iron in nutrient solution by synthetic chelating agents (e.g., EDTA) facilitates its uptake by roots and transport to the leaves. Thus, Jacobson (1951) was able to maintain a satisfactory supply of available iron to plants by a single addition of K-EDTA (5-10 ppm) to the nutrient solution (pH 5.5-7.5), in which the plants were grown for 6-8 weeks. Moreover, autoradiographs of soybeans grown on a calcareous Millville soil (pH 7.9) containing diethylene-triamine-pentaacetate (DTPA) chelate labelled with ^{14}C in the carboxyl group showed the presence of radiocarbon in the plant leaves (Holmes & Brown, 1955). All these observations indicate that iron chelates may be absorbed by

the roots and transported to the upper parts of the plant.

Growing roots are known to release appreciable amounts of organic acids, amino acids, and phenolic compounds. In many dicotyledonous species, iron deficiency leads to an increase in the release of phenolics from the roots. Some of these phenolic compounds enhance the mobilization of iron in the rhizosphere by chelation. Organic acids, such as malate, can also act as iron chelating agents and may facilitate iron uptake from the growth medium (Marschner, 1986).

Fixation of CO_2 / HCO_3^- into organic acids, mainly malate, in roots is well documented (Poel, 1953; Jackson & Coleman, 1959; Bedri et al., 1960; Splittstoesser, 1966; Jacoby & Laties, 1971; Popp *et al.*, 1982; Vapaavuori & Pelkonen, 1985). It might be expected, therefore, that plants in which the CO_2 fixing capacity of the roots increases markedly in response to elevated CO_2 or HCO_3^- would be most resistant to chlorosis, due to the additional availability of organic acids for chelation of Fe. However, studies of calcifuge species, in which there is substantial organic acid synthesis in the roots when supplied with HCO_3^-, suggest that sequestration of iron in the vacuoles by elevated levels of organic acids, can inhibit iron transport to the shoot and thus cause iron chlorosis (Lee & Woolhouse, 1969_b). A similar process could happen in the leaf cells since leaves are known to contain substantial amounts of organic acids. This could reduce the availability of iron for metabolism and consequently increase Fe-chlorosis. It is worth noting in this context, that DeKock *et al.* (1979) and Mengel *et al.* (1984) found that it is the HCl-soluble fraction of iron and not total iron that is reduced in chlorotic leaves. This fraction was termed "active iron" by Oserkosky (1933) as it was thought to participate in chlorophyll formation.

2.1.2.3. Interactions between [CO_2] and cellular pH.

Although some researchers (Brown & Wadleigh, 1955; Porter & Thorne, 1955) have claimed that HCO_3^- acts independently of pH changes in inducing chlorosis,

consideration of the Henderson-Hasselbach equation shows that the CO_2 / HCO_3^- ratio can not be changed without a simultaneous change in pH values. Experimental evidence of the way in which changes of CO_2 affect soil pH was obtained by Whitney & Gardner (1943) who showed that soil pH is approximately a straight-line function of the logarithm of the soil CO_2 in the range 0.03% to 100% CO_2 at constant moisture.

Changes in CO_2 levels in the medium surrounding the cells may also affect intracellular pH if the CO_2 change affects intracellular concentrations of CO_2 and HCO_3^- (Bown, 1985). Early experiments by Jacobs (1922) provided clear evidence that CO_2 enters animal cells passively. This author exposed starfish eggs, stained with neutral red, to solutions of pH 7.2 but containing CO_2 / HCO_3^- in one case and NH_3 / NH_4^+ in the other. The cytoplasm of the egg cells turned acid or alkaline, respectively. This experiment suggested that the neutral species of CO_2 and NH_3 moved passively, on account of their lipid solubility, through cell membranes and that the intracellular pH changed following hydration of CO_2 ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$) and NH_3 ($NH_3 + H_2O \rightarrow NH_4^+ + OH^-$). Furthermore, DeWeer (1978) found that exposing a squid axon at constant extracellular pH (slightly alkaline) to sea-water in equilibrium with 5% CO_2 resulted in a sharp drop in the intracellular pH which rose again, after removal of CO_2, to a value higher than the initial one. Somewhat similar results were reported by Kurdjian *et al.* (1978) who also showed that exposure of *Acer pseudoplatanus* cells to 5% CO_2 resulted in a decrease in intracellular pH averaging 0.6 unit. After termination of CO_2 exposure, the intracellular pH rose to a value higher than the initial one. The authors concluded that these cultured plant cells behaved similarly to animal cells with respect to passive inward diffusion of CO_2 and resulting changes in intracellular pH. Support for their conclusion came from Kolesch *et al.* (1984) who found that changes in the cytoplasmic pH of sunflower leaf cells were primarily due to additions of HCO_3^- to the medium rather than high pH values of the nutrient medium.

The relationship between varying HCO_3^- / CO_2 molarity ratio and the pH, calculated from the Henderson-Hasselbach equation is shown in the following Table:

CO_2, %	$[HCO_3^-]$ in solution at different pH, mmol m^{-3}.				
	4.5	5.5	6.3	7.5	8.0
0.033	0.155	1.55	9.77	155	490
1.5	7.14	71.4	450	7144	22590
3	14.28	142.9	900	14290	35890
5	23.25	235.5	1480	23500	75000

N.B. In water equilibrated with air at 1 atmospheric pressure and 25 °C, the concentrations of the CO_2 and H_2CO_3 are 11.1 mmol m^{-3} and 0.029 mmol m^{-3}, respectively (Bown, 1985).

Although the specific effects of any changes in intracellular pH on the metabolic processes are not clear, it is expected that even small changes in intracellular pH will influence the activities of many enzymes, which are very sensitive to changes in pH, such as phospho-enol-pyruvate carboxylase (PEPc) which plays a major role in the dark fixation of CO_2 by plant roots. It should be noted in this context that Hiatt (1967) demonstrated that changes in levels of organic acids in barley roots were proportional to expressed sap pH changes induced by unbalanced ion uptake. He suggested that the carboxylation of PEP to form C_4-organic acids and the breakdown of these acids may be regulated by the pH of the cell sap. He also confirmed earlier reports (Ulrich, 1941-42; Jacobson, 1955; Jackson & Coleman, 1959) that the organic acid content of roots increases when cations are absorbed in excess of anions.

However, despite various metabolic events that could change pH, the cytosol pH of higher plant cells is thought to be maintained within narrow limits (Davies, 1973, 1986; Smith & Raven, 1979). Davies (1973, 1986) has suggested that PEPc may function in conjunction with malic enzyme as a pH-stat for regulating intracellular pH.

$$PEP + HCO_3^- \xrightarrow[PEPc]{\text{[high pH]}} OAA \xrightarrow[MDH]{} malate \xrightarrow[\text{malic enzyme}]{\text{[low pH]}} pyruvate + CO_2$$

Where: PEPc = phospho-enol-pyruvate carboxylase; OAA = oxaloacetate and MDH = malate dehydrogenase.

According to this scheme, an increase in cytoplasmic pH (efflux of H^+ in exchange for K^+ ions) would lead to an increase in carboxylation by increasing the activity of PEPc, thereby leading to the formation of malic acid and thus returning the pH near the balance point. On the other hand, a decrease in intracellular pH (accumulation of malate and excess H^+ generation) would inhibit the PEPc and activate malic enzyme, so that pyruvate and CO_2 are formed, thereby returning the pH near the balance point.

The kinetic properties of PEPc are consistent with a role in the pH-stat; (a) PEPc has a pH optimum between 7.0 and 8.0 (Bonugli & Davies, 1977; Hill & Bown, 1978; Stout & Cleland, 1978); (b) increases of 0.1 to 0.6 units in pH, within the range 7.0 to 7.6, resulted in two to four-fold increase in saturated or PEP limited PEPc activity (Hill & Bown, 1978; Smith *et al.*, 1979; Mathiew, 1982-83), and (c) inhibition of PEPc activity by malate was reduced as pH was raised above 7.1 (Smith *et al.*, 1979; Wedding *et al.*, 1990).

Several other *in vitro* experimental data, supporting the concept of a cellular pH-stat, have been obtained. For example; enhanced rates of dark fixation of CO_2 / HCO_3^-, leading primarily to malate synthesis, have been shown to be accompanied by H^+ - K^+ exchange in root tissues (Van Steveninck, 1966; Jacoby & Laties, 1971); guard cells of *Vicia faba* (Allaway, 1973; Raschke & Humble, 1973); and *Avena* coleoptiles (Haschke & Lüttge, 1975,1977; Johnson & Rayle, 1976; Stout *et al.*, 1978; Cleland 1976). Furthermore, the inhibition of H^+ efflux when suspensions

of *Asparagus* mesophyll cells were switched from bubbling with air to CO_2-free air also demonstrates that CO_2 fixation is involved in H^+ efflux (Bown, 1982).

Others have questioned the validity of the pH-stat. For example; Roberts *et al.* (1981), using nuclear magnetic resonance (NMR) to measure intracellular pH, found that when maize roots were incubated in 25 mM K_2SO_4 to produce the maximum efflux of H^+ (K^+ is a rapidly absorbed ion that exchanges with H^+ whereas SO_4^{-2} is a slowly absorbed ion), no change in cytosolic pH could be detected and they argued that this observation ruled out the biochemical pH-stat. However, Davies (1986) pointed out that Roberts *et al.* (1981) had made their NMR measurements at a very low temperature (5 °C), and since Aducci *et al.* (1982) have noted a marked effect of temperature on cytosolic pH, their argument is questionable.

2.1.3. Effects of CO_2 on growth of root-nodulated plants.

Mulder & Van Veen (1960) were the first to report the effect of CO_2 concentration in the rooting medium on nodulation, N_2-fixation, and growth of nodulated legumes grown in a nutrient solution aerated with either air containing 3-5% CO_2 or CO_2-free air. In their first experiment, they found that inclusion of 4% CO_2 in the aeration stream, supplied to roots of red clover (*Trifolium pratense*) increased the dry matter production of the tops by 80%, the nitrogen content of the tops and the roots by 10% and 30%, respectively, and the total amount of nitrogen fixed per plant by 150%. They also noticed that in the absence of CO_2 the roots were thicker but considerably less branched and the nodules were smaller and fewer in numbers. Shoot development was also poorer and the leaves were lighter green in colour when CO_2 was absent from the aeration stream. Similar results were obtained in a second experiment in which they claimed that the presence of CO_2 in the nutrient solution was more important for red clover growth during the first part (5 weeks in this case) of the growing period than during the second (2½ weeks). In another experiment with pea (*Pisum sativum*) and bean (*Phaseolus vulgaris*) plants, they found that nitrogen fixation by pea nodules was increased considerably and the fresh weight of the nodules was about 20% greater when 3-5% CO_2 was supplied to the water culture, compared with plants aerated with CO_2-free air. In the case of the bean plants, the number of nodules was much higher and both nitrogen fixation and growth were increased when CO_2 was present in the aeration stream.

In each experiment they compared the effects of CO_2 at two pH levels (4.8 and 6.4) and found that the positive effects of CO_2 were more pronounced at a pH of 4.8. Consideration of the Henderson-Hasselbach equation shows that at this pH (4.8) most of the dissolved carbon is in the form of CO_2. Furthermore, Jarvis (1971) reported that at pH 5 about 96% of the dissolved CO_2 is in the form of CO_2, but at pH 8 HCO_3^- is the main (97.2%) component. Therefore, if the positive effect of

increased CO_2 at this pH (i.e 4.8) was due to enhancement of carboxylation activity in the nodules, it is clear that CO_2 that crossed the cellular membranes *via* inward diffusion must be protonated within the cytoplasm to produce HCO_3^- as substrate for carboxylation reactions.

In contrast to Mulder and Van Veen's results, Grobbellar *et al* (1971) did not observe any special requirement for CO_2 for nodulation of cultured roots of *Phaseolus vulgaris*, but inclusion of 4% CO_2 in the aeration supply to the culture medium completely inhibited nodulation. The authors suggested that the former observation could be due to the fact that a sufficient concentration of CO_2, resulting from respiration, was available to the explants and the bacteria since they were kept enclosed in petri dishes containing a culture solution with a pH of 6.8.

2.1.3.1. Enzymes of dark CO_2 fixation.

Since it's first discovery by Bandurski & Greiner (1953) in extracts of spinach leaves, PEPc has been shown to be widely distributed in both non-photosynthetic and photosynthetic tissues of higher plants. Several studies carried out in the 1950's and 1960's (e.g., Bandurski, 1955; Tchen & Vennesland, 1955; Walker & Brown, 1957; Ting & Dugger, 1967) have demonstrated clearly that the PEPc has a high affinity for CO_2 and is the primary enzyme responsible for CO_2 fixation in these tissues by catalysing the reaction between phospho-enol-pyruvate and CO_2 . In the last 15 years, the presence of PEPc in legume nodules at activities higher than those found in roots and its role in CO_2 fixation have been well established (Lawrie & Wheeler, 1975; Christeller *et al*, 1977; Duke *et al.*, 1979; De Vries *et al.*, 1980; Groat *et al.*, 1984; Smith, 1985). Most studies showed that much of this PEPc activity is associated with host plant cell cytoplasm, but there has been controversy concerning the occurrence and expression of activity of this enzyme in bacteroids. A requirement of CO_2 for growth of several *Rhizobium* species (*R. trifolii, R. phaseoli, R. leguminosarum, R. japonicum*) was shown by Lowe & Evans (1962).

These authors claimed that bacteroid extracts isolated from soybean nodules contain very active PEPc and propionyl CoA. carboxylase enzymes. In contrast, Christeller *et al.* (1977) found that although the PEPc activity in the bacteroid-containing zone was 4 to 6 times greater than that detected in the cortex tissue, when bacteroids were purified from nodule homogenates they had no detectable PEPc activity and most of the activity was recovered in the plant cytoplasmic fractions. Furthermore, recent data derived from immunochemistry have confirmed that the nodule PEPc is of plant rather than bacterial origin (Perrot-Rechenmann *et al.*, 1981; Vidal *et al.*, 1986; Miller *et al.*, 1987; Suganuma *et al.*, 1987).

While the major part of CO_2 fixation in root-nodulated legumes is catalysed by PEPc, in the actinorhizal plant *Alnus glutinosa* CO_2 fixation is catalysed by both PEPc, which also shows greater activity in nodules than in roots and leaves (Perrot-Rechenmann *et al.*, 1981) and carbamyl phosphate synthetase (CaPs) (McClure *et al.*, 1983). The immunochemistry studies cited above have also shown that CaPs in *Alnus* and PEPc in both *Alnus* and legume nodules are located in the cytosol.

2.1.3.2. The role of dark CO_2 fixation in N_2 fixation and N assimilation.

Mulder & Van Veen (1960) attributed the increase in symbiotically-fixed nitrogen in leguminous plants, which were grown in culture solution supplied with CO_2, to a more liberal supply of α-keto acids, which were found to occur in the nodules of these plants, compared to plants grown in solutions aerated with CO_2-free air.

Confirmation of their interpretation was not possible at that time due to the lack of an easy assay to study the relationship between dark CO_2 fixation and nitrogen fixation. However, later studies (reviewed by Hardy *et al.* 1973) showed that acetylene is reduced to ethylene by nitrogen-fixing systems *via* nitrogenase activity and that acetylene reduction by nitrogenase has the same requirements for reductant and ATP as does the reduction of nitrogen to ammonia. These

observations led to many investigations, in which the acetylene reduction method was used as a sensitive assay for nitrogen fixation and for studying its relationship with dark CO_2 fixation in different plant materials (eg., detached nodules, decapitated intact root systems or the root systems of intact plants). These investigations provided a great deal of support to Mulder & Van Veen's interpretation by demonstrating that the dark CO_2 fixation and nitrogenase activity of nodulated legumes and of alders are linked.

Lawrie & Wheeler (1975) were the first to demonstrate a relationship between nodule CO_2-fixation by PEPc and nitrogen fixation activity for legumes when they found that increases in radioactivity were initially associated with glutamate and aspartate and later with asparagine after exposure of excised nodules of *Vicia faba* to $^{14}CO_2$. They concluded from their data that suitable acceptor molecules derived from CO_2 fixation by the nodules can provide carbon skeletons for ammonia assimilation (the root and nodule enzymes for ammonia assimilation are also located in the cytoplasm; Robertson *et al.*, 1975; Miflin & Lea, 1976; Scott *et al.*, 1976). Two years later Christeller *et al.* (1977) provided clear evidence in support of this conclusion when they demonstrated significant correlation between nodule acetylene reduction (a measure of nitrogen fixation) and both *in vivo* CO_2 fixation and *in vitro* PEPc activity during nodule development in lupin. Further support for Lawrie and Wheeler's conclusion include: (a) in alfafa nodules both *in vitro* PEPc activity (Vance *et al.*, 1983; Maxwell *et al.*, 1984) and glutamate synthase activity (GOGAT) (Vance *et al.*, 1983; Groat *et al.*, 1984) parallel changes in acetylene reduction activity; (b) inefficiency of N_2-fixation was accompanied by low *in vitro* PEPc activity (Deroche *et al.*, 1983; Rosendahl & Jakobsen, 1987; Rosendahl *et al.*, 1990); and (c) treatments that inhibit nitrogen fixation caused a parallel reduction in CO_2-fixation (Laing *et al.*, 1979; Vance *et al.*, 1983; Anderson *et al.*, 1987). By contrast, Coker & Schubert (1981) reported that during the vegetative stages of growth of nodulated soybeans, reliant on fixed N as sole source

for growth, CO_2 fixation in nodules increased at the onset of N_2 fixation but declined to a lower level prior to the decrease in N_2 fixation. This decline coincided with a decrease in the transport of amino acids, especially asparagine, and an increase in the export of ureides.

Vance *et al.* (1985) compared the partitioning and transport of $^{14}CO_2$ fixed by nodules of alfafa on the one hand and soybeans and adzuki bean on the other. In agreement with earlier data reported by Maxwell *et al.* (1984) which showed that $^{14}CO_2$ fixed by nodules of alfafa and birdsfoot trefoil was transported as aspartate and asparagine in xylem sap into the shoots, they found that radioactivity in the xylem sap of nodulated alfafa was primarily in amino acids with only about 20% in organic acids (removal of nodules resulted in a 99% decrease in xylem sap radioactivity). In soybean and adzuki bean, however, radioactivity was located primarily (70-87%) in TCA cycle intermediates (acid fraction), which is consistent with earlier suggestions that TCA cycle acids synthesized in nodules and roots of ureide transporters are transported to balance excess inorganic acid cation charge of the xylem sap (Israel & Jackson, 1982). Only about 10 to 22% of the total radioactivity in xylem sap was located in the amino acid fraction (mainly ureides); which suggests that nodule CO_2 fixation in soybeans and adzuki beans contribute little of the carbon required for assimilation and transport of fixed nitrogen.

It appears, therefore, that the transported form of fixed nitrogen in legumes may determine the nature of the relationship between nodule CO_2 fixation activity and N_2 fixation activity. On the one hand, the two processes appear to be positively correlated in the amide (mainly asparagine, ASP; and glutamine, GLN) transporting legumes such as, alfafa, lupin and peas. In these species, oxaloacetate produced by PEPc activity is metabolized into glutamate, aspartate, and asparagine, which is normally the main aminated compound exported from the nodules (Pate & Wallace, 1964). On the other hand, in the ureide (allantoin, ALN; and allantoic, ALC) transporting legumes such as soybean, a major portion of dark CO_2 fixation is

associated more closely with nitrogenase activity than with ammonia production and assimilation (King *et al.*, 1986). In these species, as suggested earlier by Coker & Schubert (1981), C_4- acids, the primary products of CO_2 fixation in nodules, may not be required for ureide synthesis.

In actinorhizal plants, the majority of species examined export the amide asparagine (Schubert, 1986). Nevertheless, citrulline (a ureide amino acid) has been shown to be the major soluble nitrogenous compound in nodules, roots and leaves (Miettinen & Virtanen, 1952), in xylem exudate and nodule extracts (Schubert *et al.*, 1981) and in root pressure sap and stem sap (Tonin *et al.*, 1990) of *Alnus glutinosa.* Similar results were reported in xylem sap of *Casuarina equisetifolia* (Walsh *et al.*, 1984). However, it was Gardner & Leaf (1960) who first demonstrated incorporation of ^{14}C, supplied as $H^{14}CO_3^-$ to the roots of water solution-grown *Alnus glutinosa* , into organic and amino acids. After 4 hours, citrulline was the only amino acid to contain ^{14}C in the leaves, although glutamic acid in the stem also showed some activity. To examine the role of CO_2 fixation in the process of nitrogen fixation and synthesis of citrulline in *A. glutinosa*, McClure *et al.* (1983) extended the studies of Gardner & Leaf (1960). They found that radioactivity was incorporated into only three amino acids, glutamate, aspartate and citrulline, after incubation of detached nodules with $^{14}CO_2$ for periods up to 40 min. These amino acids incorporated 10%, 8% and about 40% of the total radioactivity, respectively. Investigation of the position of labelling of citrulline revealed that approximately 80% to 90% of the ^{14}C incorporated was in the carbamyl group, with the remaining 10 to 20% in C_1 position presumably derived *via* glutamate from $^{14}CO_2$ fixed by PEPc. From these data they concluded that fixation by CaPs is, therefore, the principal route by which CO_2 is utilized for citrulline synthesis in alder, although it only accounts for about 30 to 40% of the total $^{14}CO_2$ fixed. The majority of CO_2 fixation is catalysed by PEPc but a major portion of the C_4-acids produced contribute little of the ^{14}C required for assimilation and transport of fixed

nitrogen. These data are in agreement with those shown for ureide transporting legumes (Coker & Schubert, 1981; Vance *et al.*, 1985; and King *et al.*, 1986).

McClure *et al.* (1983) also demonstrated that the major labelled organic acid in nodule extracts was malate with lesser amounts of ^{14}C in citrate and succinate. The time course of labelling of organic and amino acids and pulse-labelling experiments showed that: (a) the incorporation of ^{14}C into malate continued to increase at a linear rate for 40 min., whereas the incorporation of ^{14}C into citrate decreased, (b) after the initial decrease in the percentage of total ^{14}C in organic acids (corresponding to an increase in the labelling of amino acids), there was no change in the distribution of ^{14}C between the two fractions throughout the remainder of the time course experiment and (c) the percentage of the total label in the organic acid and amino acid fraction was unchanged after pulse-labelling, indicating that there is little exchange between the organic acid pool and the amino acid pool. From these data the authors concluded that malate in the nodules was present in two main "pools". One pool is rapidly converted into citrate and subsequently used to synthesise glutamate. The second, the largest, appeared to be metabolically inactive during the incubation of detached nodules. In intact plants, however, they suggested that this accumulated malate could be exported from the nodules as a counterion for cation transport in xylem as suggested previously for soybeans (Coker & Schubert, 1981) or it could be involved in maintaining celullar pH balance, as discussed before.

Estimates of the magnitude of CO_2 fixation suggest that it is of a similar order in legume and actinorhizal nodules. In soybean nodules, active in N_2 fixation, the amount of CO_2 fixed is about 14% of that released during nodule respiration (King *et al.*, 1986). In alder nodules, McClure *et al.* (1983) found that detached nodules of *Alnus glutinosa*, reducing 13.5 µmol C_2H_2, fixed 8.8 µmol CO_2 (µg dry wt)$^{-1}$. h^{-1}. The data of Tjepkema & Winship (1980) for detached nodules of *Alnus rugosa* show an average ratio of 3.4 between rates of respiratory CO_2 evolution and

C_2H_2 reduction. Using both setes of data it can be calculated that in alders respiration may provide about 19% of the CO_2 fixed in the nodules. Some 80-85% of the substantial input of carbon fixed in nodules active in N_2 fixation thus is provided by uptake of HCO_3^- and/or by passive inward diffusion of CO_2.

Fixation also occurs in the roots per se but at a much lower rate than in the nodules [the activity of alder nodules (g. dwt.)$^{-1}$ was about 4 times that of the roots; McClure *et al.*, 1983]. However, because nodules normally form 5% or less of the dry weight of the root system, CO_2 fixation by the roots alone must constitute a major source of fixed carbon. Thus, Anderson *et al.* (1987) showed that nodules of alfafa fixed CO_2 at a rate of 5.6 times that of roots; but since the nodules comprised a small portion of root system mass (about 4%), the roots actually accounted for 76% of the nodulated root system CO_2 fixation. Despite this, Anderson *et al.* (1987) noted a dramatical drop of total radioactivity of the amino acid fraction in xylem sap of denodulated roots compared with nodulated roots, which suggests that $^{14}CO_2$ fixed by roots contribute little to assimilation and transport of fixed nitrogen (similar results were reported for xylem transport compounds in ineffectively nodulated roots compared with effectively nodulated roots of alfafa, Maxwell *et al.*, 1984). The labelled compounds in the xylem sap of denodulated roots were mainly organic acids, which may serve as counterions to balance cation transport (Gunning *et al., 1*974; Israel & Jackson, 1982)

The metabolism of fixed CO_2 in *Alnus* nodules is thus quite well documented. However, the effects of changes in CO_2 levels on growth have not been reported. It is this latter aspect which has been studied here.

2.2. MATERIALS AND METHODS.

2.2.1. Germination of seeds.

Alnus glutinosa seeds were purchased from the Forestry Commission (Farnham, Surrey) and stored in a cold room at 2 °C prior to use.

Seeds were germinated in seed trays in perlite moistened with a nutrient solution consisting of Crones-N, 0.5 g and Hoaglands A-Z micro-nutrients, 0.2 ml in 2 litres of distilled water (for composition of nutrients see Hooker, 1987). Standard-grade perlite was moistened with the nutrient solution and mixed thoroughly before being layered in the trays; the pH was adjusted to 6.2 with 2 N sulphuric acid. The seeds were spread on top of the standard-grade perlite and covered with a thin layer of super-fine grade perlite. The trays, covered with clear plastic domes, were maintained in a controlled environment growth cabinet (photoperiod 16 hours; temperature 21°C light, 19°C dark) illuminated by warm-white fluorescent tubes (33 W m^{-2}).

2.2.2. Seedling inoculation and plant growth.

2.2.2.1. Perlite-grown seedlings.

About forty days after sowing, that is, after the emergence of the first true leaf but before the emergence of the second, the seedlings were carefully transplanted into 1¼ L-capacity black plastic bags, filled previously with perlite perlite moistened with a nutrient solution consisting of Crones-N, 1.0 g and Hoaglands A-Z micro-nutrients, 1 ml in 2 litres of distilled water. The bags, containing three seedlings each, were stood in groups within seed trays in a heated greenhouse supplemented with 400 W mercury vapour lamps (16 hours photoperiod, above 15°C).

A week later, *Frankia* UGL 010708 stock cultures were harvested by

filtration on 0.2 µm membrane filter (Millipore, 47 µm in diameter) "*in vacuo*" using a millipore unit. The residue was rinsed several times with distiled water, resuspended in distilled water and homogenised thoroughly in a Potter-Elvehjem homogeniser. The final homogenate was used for inoculating the seedlings by forcing equal quantities of the *Frankia* homogenate through a syringe fitted with a needle (gauge, 21), over the perlite surface at the base of the shoot of each seedling. During the first 2 weeks after inoculation, the seedlings were given maintenance doses of combined N in the form of Fision's Liquinure (Hooker, 1987). Inoculated seedlings were maintained in a heated greenhouse as before and watered regularly with tap-water.

2.2.2.2. Water culture-grown seedlings.

Again about forty days after sowing, that is, after the emergence of the first true leaf but before the emergence of the second, the seedlings were carefully transferred to 2 l-capacity pots filled with a nutrient solution consisting of distilled water containing Crones-N, 0.5 g and Hoaglands A-Z micro-nutrients 0.2 ml. Seven seedlings were positioned in each pot and were held in place, within holes drilled in the black "Perspex" lid, with a ring of rubber tubing and cotton wool. The pots were arranged randomly within a controlled environment growth cabinet (for conditions see 2.2.1).

Two weeks later, the nutrient solution was replaced with a fresh solution containing Crones-N, 1.0 g and Hoaglands A-Z micro-nutrients 1 ml dissolved in 2 litres of distilled water. Each pot was inoculated by forcing equal quantities of *Frankia* UGL 010708 homogenate (prepared as before) through a syringe, over the entire surface of the solution. During the first 2 weeks after inoculation, the seedlings were given maintenance doses of combined N as above. The pots were maintained in a heated greenhouse as before and topped up regularly with tap-water.

2.2.3. Nitrogenase (C_2H_2 reduction) activity and root respiration.

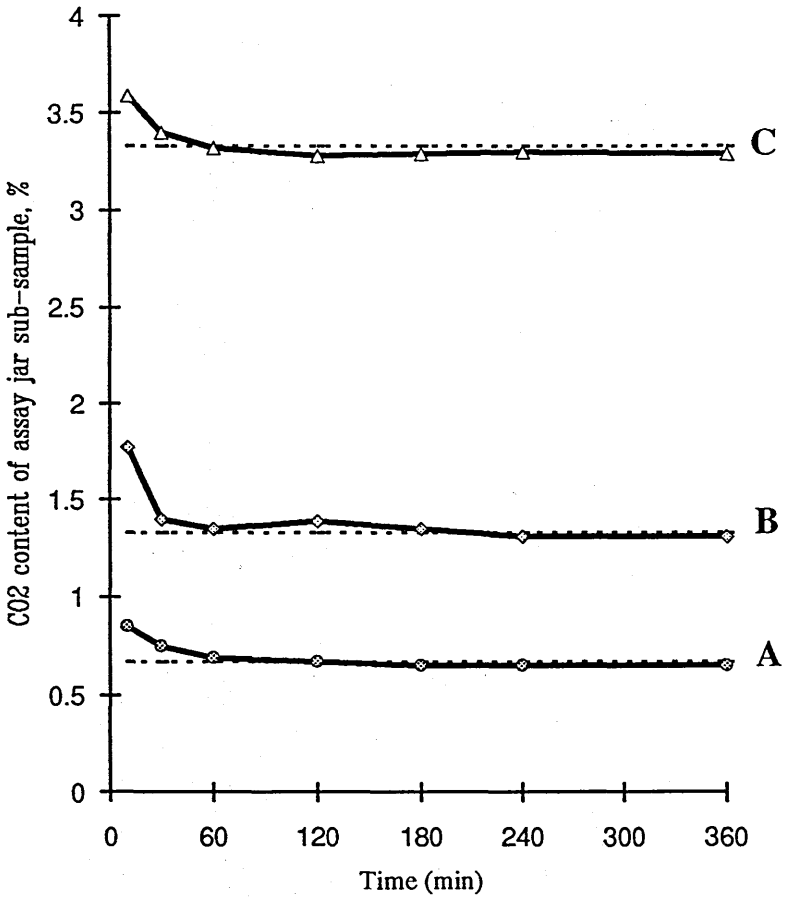
2.2.3.1. Effect of CO_2 on C_2H_2 reduction of intact plant root systems.

For this experiment 18 plants were selected for uniformity in size from those plants which had been grown in perlite for 16 weeks as described in 2.2.2.1.

On the day before the experiment started, the potted root systems of intact plants were placed inside 1.4-L glass jars (one plant per jar). The following morning, each jar was closed with a split two-hole plastic lid. The two halves of the lid were positioned around the base of the plant stem, which passed through the central hole, and then sealed firmly to each other with "Vaseline". The sealing operation was completed by molding "Plasticine" along the split line and around edges of the lid. The second hole on the lid was fitted with a rubber stopper to facilitate gas injection.

As soon as the jars were sealed 10 % (v/v) of the air in each incubation jar was withdrawn with a syringe and replaced with acetylene. Triplicate 0.5 ml gas samples were sampled from each jar hourly and assayed for ethylene and acetylene concentrations, using a Pye Unicam Series 104 Gas Chromatograph equiped with a 1 metre x ¼ inch steel column of Poropack N (100-120 mesh). The chromatograph was operated isothermally (60 °C), with a carrier gas of oxygen-free nitrogen (flow rate, 35 ml.min-1) and flame ionisation detector (hydrogen/air flame). After 3 hours, that is immediately after the third triplicates were sampled, different amounts of CO_2 were injected into 15 of the original 18 jars (amounts added are shown in Figure 2); the remaining three jars which did not receive any additional CO_2 served as controls. After incubation for one hour the sampling was repeated as before for 3 hours. A preliminary experiment showed that when CO_2 is injected into the acetylene assay jars it reaches full equilibrium with the air already present in the jars within 30 to 60 min of injection (Figure 1).

Figure 1: Equilibration of CO_2 injected into acetylene reduction assay jars.[1]



[1] Three assay jars containing a quantity of moistened perlite, equivalent in volume to the transpots in which the experimental plants were grown, were sealed tightly as described before . Known amounts of CO_2 (5 ml, 10 ml and 25 ml) were then added by syringe to the closed jars, A, B, and C, respectively, and changes in the CO_2 content of triplicate 0.5 ml samples from each jar, sampled at different intervals over a period of 6 hours, were assayed by thermal conductivity gas chromatography, using a Pye Unicam Series 104 Gas Chromatograph equiped with a 1 m x 6 mm steel column of Porapak R (100-120 mesh). The chromatograph was operated isothermally (55 °C), with a carrier gas of helium (flow rate, 50 ml.min^{-1}) and thermal conductivity detector (250 mA). When these added volumes of CO_2 reach full equilibrium with the air enclosed within the jars they should correspond to calculated CO_2 concentrations of: A, 0.67%; B, 1.33% and C, 3.33% of the assay jar gas-phase as shown by the dotted lines on the graph.

During the acetylene assay the jars were kept in a controlled environment growth cabinet (see 2.2.1 for conditions).

After completion of the acetylene assay, the plants were removed from the

jars and the root systems were freed from the potting mixtures by shaking followed by washing with tap-water. The nodules were picked carefully then dried to constant weight at 80°C for 24 hours.

Ethylene evolution was corrected for leaks, as evaluated by changes in acetylene content. Quantification of detector response was by comparison with the peak height given by a standard gas (95 ppm ethylene in argon, British Oxygen Company Special Gases). Corrections were made for temperature, sampling, relevant jar volume and nodule dry weights.

2.2.3.2. Respiratory CO_2 evolution by nodulated roots of intact plants.

In order to know what changes in the CO_2 content of the rooting system gas were likely to occur during the experimental period of experiment (2.2.3.1.), respiratory evolution of CO_2 by nodulated roots of intact *A. glutinosa* contained in assay jars was measured in a separate experiment where neither C_2H_2 nor CO_2 was added to the assay jars. For this, three intact nodulated *A . glutinosa* plants of the same age as those used in experiment 2.2.3.1., were enclosed in the assay jars as described before. The jars were then incubated in the growth cabinet and triplicate 0.5 ml samples from each jar were sampled hourly, over a 6 hour period, and assayed for CO_2 content by thermal conductivity gas chromatography.

2.2.4. Effect of CO_2 levels in the root system aeration supply on N_2-fixation and growth of water-culture grown *A. glutinosa.*

2.2.4.1. Setting-up the experiments.

After 40 days of growth in water culture as described in 2.2.2.2., the plants to be used in the growth experiments were selected for uniformity in size. The plants were transferred to 2 l.-capacity glass jars filled with a nutrient solution consisting of distilled water containing Crones-N, 2.5 g; Hoaglands A-Z micro-nutrients, 2 ml; pH was adjusted to 6.3. The sparingly soluble Crones salts were wrapped in a nylon net (100 μm in diameter) to prevent deposition of salts on the root system.

Each jar was closed with a waxed cork lid bearing 5 holes, three for the plants and two for gas inlet and outlet tubes. The plants were sealed in the holes with rings of rubber tubing and cotton wool. The gas inlet hole, drilled in the centre of the jar lid was fitted with rubber tubing which was connected to the desired aeration gas source at one end and immersed in the water culture, well below the roots, at the other end. The lid was sealed to the jar with black tape. When air was bubbled through the water culture, emerging gases were piped away -well above the top of the plants- through 50 cm-long glass tube fitted to the gas outlet hole to exclude the possibility that the plant photosynthesis would be affected by the release of air containing elevated levels of CO_2. The jars were covered with black paper to keep light from reaching the rooting systems. Sealing was completed with plasticine as necessary.

2.2.4.2. Supplementation of gas supply to root systems with CO_2.

Three experiments, each consisting of two sets of 5 jars unless otherwise indicated, were set-up as described above and were carried out at different times. After completion of sealing, a gas-flow of 75 ml.min-1, controlled with regulator valves and flow-meters, was instigated. The control jars were aerated with atmospheric air

whereas the experimental jars were aerated with CO_2-free air (experiments A) or air containing 1.5% CO_2 (experiment B) or air containing 5% CO_2 (experiment C). The latter experiment contained a further set of 5 jars which were aerated with CO_2-free air.

All gas mixtures used throughout this study were obtained in cylinders from the British Oxygen Company, Special Gases, Ltd. As a precaution a sample from the CO_2-free air cylinder was checked in the laboratory for absence of CO_2 as follows: A solution of barium hydroxide (10 %) was shaken very well then filtered into two flasks, each fitted with a side arm. One solution was aerated with air from the CO_2-free air cylinder and the other with pumped air, from the growth cabinet. The test showed that while the latter solution became cloudy the former solution remained clear, which confirmed that the air in the cylinder contained no CO_2.

The experiments were carried out in a growth cabinet (16 hours, 23 °C; and 8 hours, 19 °C). Light was provided by warm-white fluorescent tubes. In each experiment the jars of different series were positioned in parallel rows (two rows in experiments A and B; and three rows in experiment C) and the positions of these rows were swopped-over on a weekly basis to reduce, as much as possible, any localized differences in the environment conditions within the cabinet.

The jars were checked daily for gas leaks by applying a drop of soapy water to the top end of the gas-outlet tubes and inspecting for bubble formation.

The pH of each culture solution was checked twice a week and adjusted to 6.3 with HCl or NaOH. This was done by first adjusting the pH of a 50-ml sample taken out of the culture solution with graduated pipette introduced through the hole left by the removal of the gas outlet tube. The calculated volume of HCl or NaOH required to adjust the pH of the full solution was then added. After pH adjustment, the gas outlet tube was repositioned. The water cultures were changed every second week.

2.2.4.3. Plant harvest and determination of nitrogen content.

After 5-6 weeks of growth, the plants were removed from the culture solutions and separated into their main constituent parts (leaves, stem, root and nodules) which were dried to constant weights at 80 °C for 24h. Shoot heights and in some cases (experiments A & B) leaf areas were measured before transferring the plant materials to the oven for drying.

Determinations of nitrogen content were carried out on dried material from each jar. The dry material from each jar (3 plants) was combined, ground to fine powder in a mill, mixed very well and then dried for further 24 hours at 70 °C before being assayed for nitrogen content using a semi-micro Kjeldahl method as follows:

Organic matter in the sample is oxidized by concentrated sulphuric acid in the presence of a catalyst. During this digestion process nitrogen is converted quantitively to ammonium sulphate. One fifth ($^1/_5$) of the digestion mixture is then made alkaline by the addition of NaOH during steam distillation, and the ammonia released is collected in boric acid. The quantity of acid neutralized by the ammonia is determined by titration with standard HCl using a mixed indicator. From this, the amount of nitrogen in the sample can be determined as follows:

Suppose that X is the weight (in grams) of digested material.

We already know that only $^1/_5$ th of X was taken for steam distillation and that 1 ml of 0.01 N HCl contain 0.14 mg N.

(mg) in digested sample = titration value (ml) x 0.14 x 5.

$$\Rightarrow \text{N. content. } g^{-1} = \frac{\text{N (mg) in digested sample}}{\text{Wt. of digested sample (X, g).}} = \text{mg N } g^{-1}$$

2.3. RESULTS.

In this section, nitrogen content per plant unit (mg. g-1 plant d.wt.) and nitrogen content per nodule unit (mg. mg-1 nodule d.wt.) will be termed as specific nitrogen content and nodule specific activity, respectively.

2.3.1. Effect of short-term elevation of CO_2 levels in the rooting medium on C_2H_2 reduction in *Alnus glutinosa*.

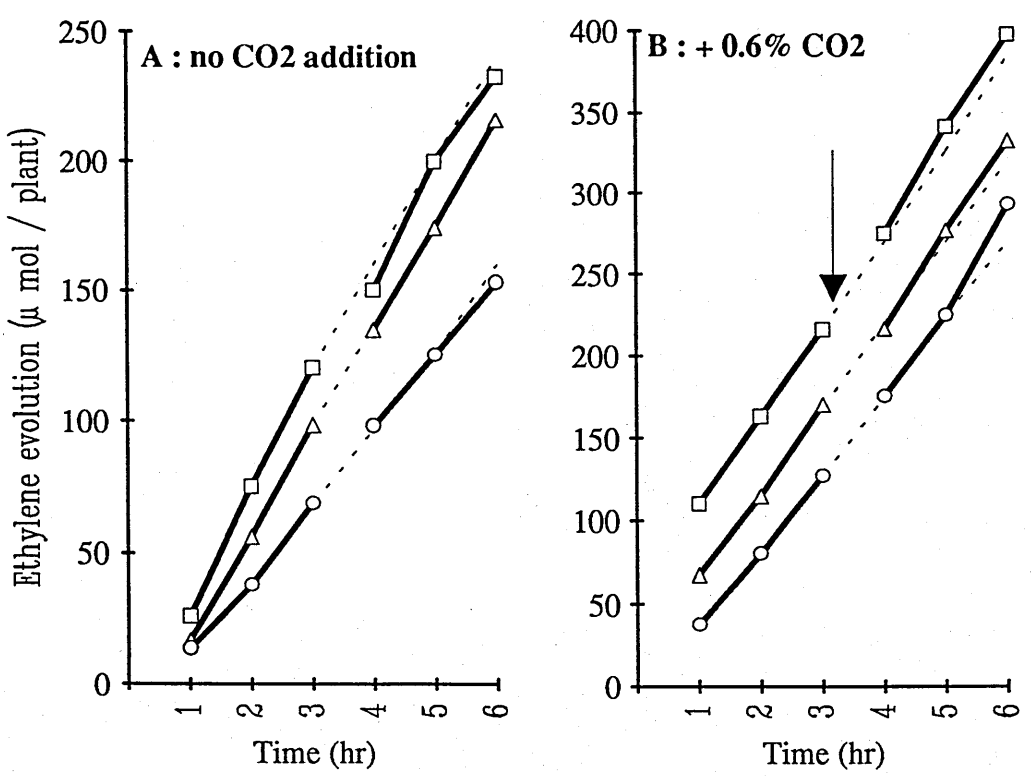
As shown in the Materials and Methods, a preliminary experiment showed that added CO_2 reaches full equilibrium with the air already present in the acetylene reduction assay jars within about 30 to 60 mins (Fig. 1). In the light of this result, it was decided that after CO_2 addition, the assay jars containing root systems would be pre-incubated for 60 mins, to allow CO_2 equilibration, before the first samples were taken out for determination of ethylene evolution rates.

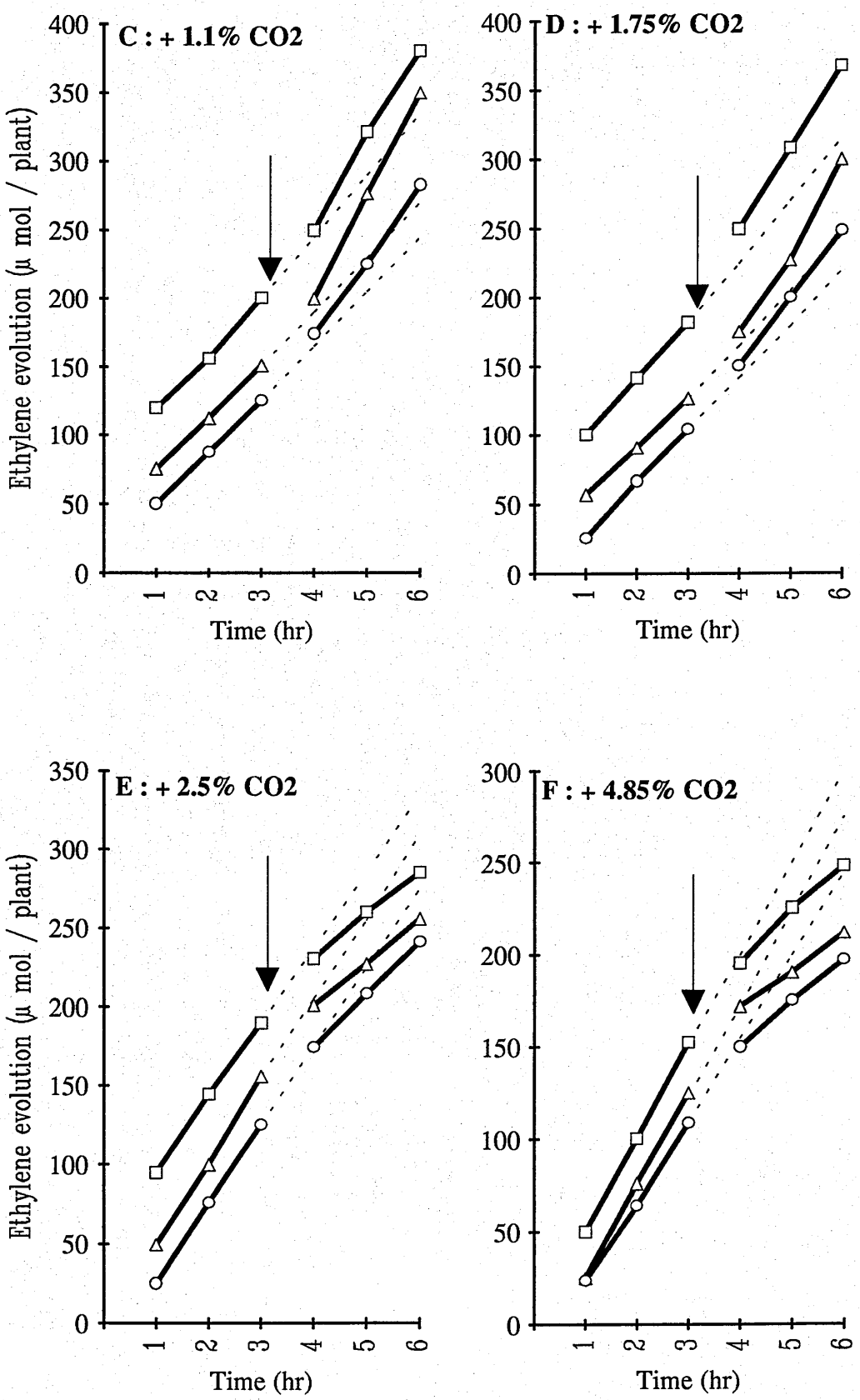
Changes in ethylene evolution rates, following elevation of CO_2 concentrations around nodulated roots of intact *Alnus glutinosa*, are plotted as a function of time in Fig. 2 (graphs, A-F). Graph A shows that ethylene evolution continued at a linear rate for 6 hrs., when CO_2 was not added to the assay jars. Changes in ethylene evolution plots with time are evident in the other graphs, following addition of CO_2 and particularly when CO_2 volumes corresponding to 1.1%, 2.5% and 4.85% of the jar gas-phase were added (graphs C, E and F).

Figure 2. Acetylene reduction (ethylene evolution) by intact nodulated *Alnus glutinosa* before and after addition of CO_2 to the root system of plants sealed in assay glass jars.

The CO_2 volumes (5, 10, 15, 20, and 40 mls) were injected by syringe into the closed jars B, C, D, E, and F, respectively. The resulting concentrations, calculated as percent of the jar gas-phase when containing intact plant root systems in perlite are shown in the individual graphs. CO_2 was added to the jars after 3 hours incubation in air and immediately after removal of sample number 3 for determination of pre-CO_2 addition ethylene evolution rates as indicated by the arrows. Results for controls where no CO_2 was added are shown in graph A.

On each graph ethylene evolution rates of 3 separate plants, subjected to the same treatment, are plotted. The dotted lines show the expected rates if the ethylene evolution continued at a linear rate for 6 hours.




C : + 1.1% CO2
D : + 1.75% CO2
E : + 2.5% CO2
F : + 4.85% CO2
Ethylene evolution (µ mol / plant)
Time (hr)
0
50
100
150
200
250
300
350
400
1
2
3
4
5
6

Rates of ethylene evolution before and after CO_2 addition are shown numerically below (Table 3) and are summarised in Figure 3 as percentage changes in the rate of ethylene evolution following addition of CO_2 to the assay jars.

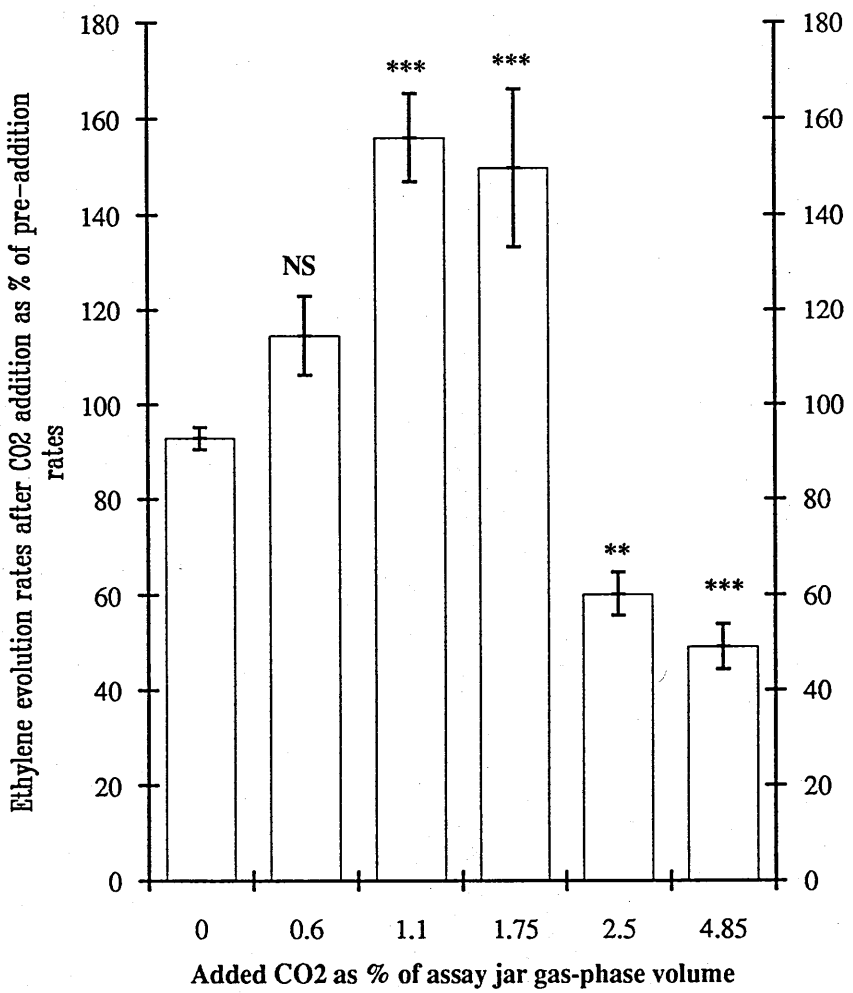
Table 3: Effect of supplying CO_2 to the root system of intact nodulated *Alnus glutinosa* on the rate of C_2H_2 reduction (ethylene evolution).[1]

Added CO_2 as % of gas-phase volume	Plant No.	C_2H_4 evolution (µmol . plant^{-1} . h^{-1}). before CO_2 addition	after CO_2 addition
Control[2]	1	46.96 (0.999)	41.51 (0.993)
	2	27.36 (0.992)	25.36 (0.999)
	3	42.22 (0.924)	40.58 (0.999)
0.6%	1	58.25 (0.998)	61.50 (0.998)
	2	54.47 (0.999)	57.74 (0.999)
	3	44.75 (0.999)	58.69 (0.995)
1.1%	1	37.60 (0.999)	54.15 (0.999)
	2	38.06 (0.999)	66.25 (0.997)
	3	43.45 (0.999)	65.30 (0.998)
1.75%	1	41.10 (1.000)	59.20 (0.991)
	2	34.75 (0.999)	62.76 (0.995)
	3	39.22 (0.999)	48.90 (0.999)
2.5%	1	53.22 (0.999)	27.42 (0.999)
	2	50.15 (0.999)	33.40 (0.999)
	3	43.00 (0.996)	26.85 (0.998)
4.85%	1	51.15 (0.999)	26.45 (0.996)
	2	42.65 (0.999)	23.75 (0.999)
	3	50.01 (0.999)	19.86 (0.998)

1. Rates of C_2H_4 evolution were calculated from linear regression analysis of data for hours 1, 2 and 3 prior to CO_2 addition, and for hours 4, 5 and 6 following CO_2 addition at the 3 hrs interval. Correlation coefficients are shown in brackets .

2. No added CO_2.

Figure 3. Percentage change in the rate of acetylene reduction (ethylene evolution) following addition of CO_2 to the root systems of *Alnus glutinosa* after 3 hours incubation in air.



Rates of C_2H_4 evolution (calculated from Table 3) represent the values in column 2 (i.e., after CO_2 addition) as % of values in column 1 (before CO_2 addition). Bars indicate, means ± standard errors (n=3) .

***, ** significantly different from the percentage change in the first bar where no CO_2 was added to the jars, at $P \leq 0.01$ and $P \leq 0.05$, respectively.

Ns, Not significantly different.

From this figure it can be seen that addition of 0.6%, 1.1% and 1.75% CO_2 apparently increased the rates of ethylene evolution by about 15%, 55% and 50%, respectively. However, only acetylene reduction rates of plants subjected to 1.1%

and 1.75% are significantly higher than those of control plants. Addition of 2.5% and 4.85% CO_2 decreased the rate by 40% and 50%, respectively.

It should be noted that the CO_2 concentrations shown in Figure 3 do not represent the total CO_2 present in the assay jars during the experiment, because they do not take into account the CO_2 evolved through respiration of the plants root systems when enclosed within the assay jars. The respiratory CO_2 evolution of 3 intact nodulated *Alnus glutinosa* plants, of the same age as those used in the previous experiment, measured in a separate experiment, set-up in the same way as the previous one but without addition of neither C_2H_2 nor CO_2 to the jars, is shown in Table 4. These data show that CO_2 evolution was linear for 6 hrs. and that the average hourly increase in jar CO_2 content due to root system respiration was 0.17 ± 0.05. From this, it is calculated that root system respiration increases CO_2 content of the assay jar gas-phase by about 0.5% after 3 hrs and 1% after 6 hrs.

Table 4: Plant part dry weights and respiratory CO_2 evolution of intact nodulated *Alnus glutinosa*.[1]

Plant	Plant part dry weights, g.		Respiratory evolution of CO_2, µmol . h^{-1}		Increase in CO_2 as % of jar gas-phase volume . h^{-1}.
	Nodules	Root system	per plant	Root system, g^{-1}	
1	0.19	1.617	87.06 (.99)	53.8	0.24 %
2	0.086	1.461	50.70 (.98)	34.7	0.14 %
3	0.092	1.562	54.40 (.99)	34.8	0.15 %

[1] Data for CO_2 respiratory evolution were calculated from linear regression analysis of the hourly CO_2 measurements. Correlation coefficients are shown in brackets. The free-gas volume in the assay jars, when containing the plant root system in perlite and measured by displacement with water, varied between 720 and 740 mls.

2.3.2. Effect of long-term changes in CO_2 levels in the root medium aeration supply on N_2 fixation and growth of water culture-grown *Alnus glutinosa.*

Results are reported for three experiments. The control jars were always aerated with atmospheric air. The experimental jars were aerated with CO_2-free air (experiment A), with air containing 1.5% CO_2 (experiment B) and with CO_2-free air or with air containing 5% CO_2 (experiment C, which involved 3 sets of jars).

2.3.2.1. Visual comparisons.

In experiment A, differences between plants aerated with CO_2-free air and plants aerated with atmospheric air were observed after about 4 weeks of growth. When CO_2 was absent from the aeration stream, the roots were less branched, the nodules were fewer in number, the shoot development was poorer, and the leaves looked less dark-green when compared with plants aerated with atmospheric air.

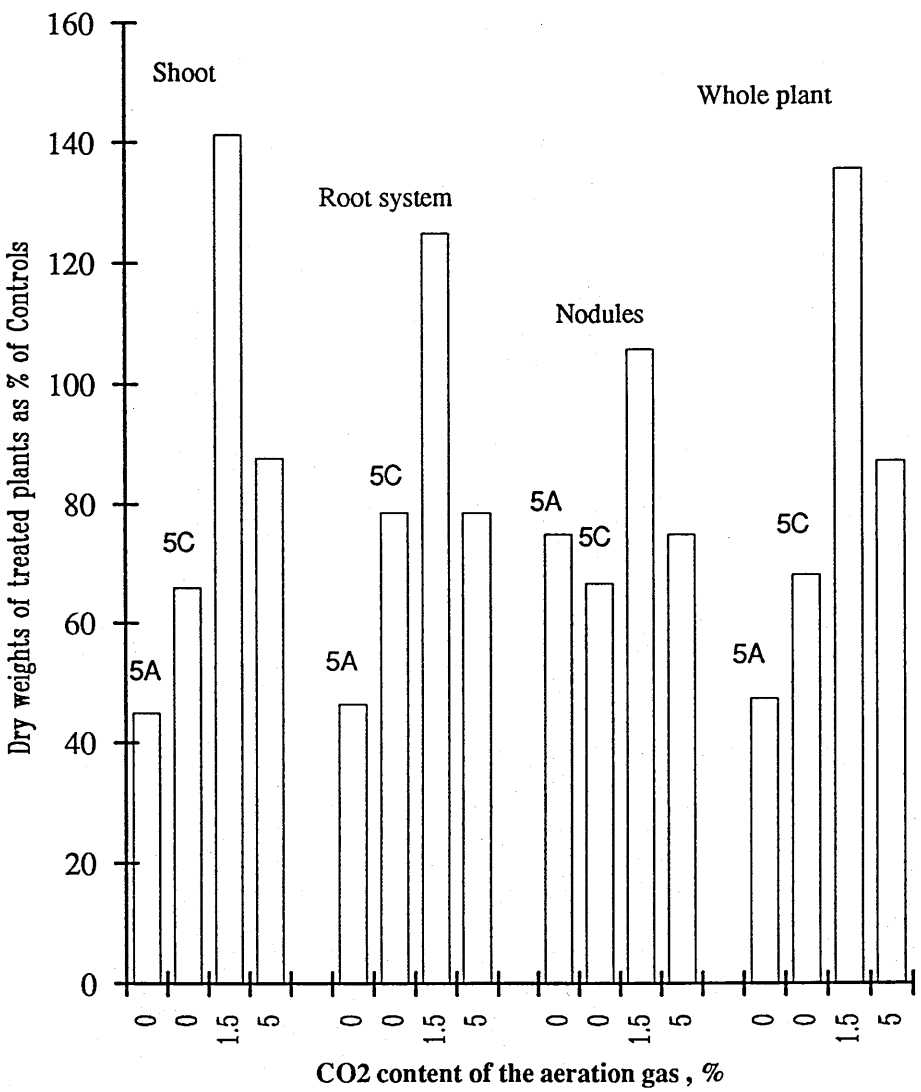
In experiment B, when 1.5% CO_2-treated plants were compared with atmospheric air-treated plants visual differences were not obvious until half-way through the third week of growth when 1.5% CO_2-treated plants appeared to be growing somewhat more strongly. Both the shoots and roots were slightly longer and the leaves were larger darker-green. The roots were more branched and had more nodules. These differences persisted for about two weeks but in the last few days before harvest they were less obvious than before.

In experiment C, the visual impression obtained after about 25 days of growth was that the plants aerated with atmospheric air looked healthier and stronger than CO_2-free air-treated and 5% CO_2-treated plants. Removal of CO_2 from the aeration stream had a negative effect on plant growth (poor development of roots and shoots, less nodules and smaller leaves). However, these differences were less pronounced than those recorded in experiment A. Inclusion of 5% CO_2 in the aeration stream also had negative but very small on root, shoot and nodules development, compared to those of controls. These differences persisted until harvest.

2.3.2.2. Comparison of dry weight accretion.

Data for plant dry matter production and their statistical analysis are shown in tables 5A, 5B and 5C, while comparisons between dry weights of treated plants and controls are summarised in figure 4.

Figure 4: Dry weight accretion of *Alnus glutinosa* plants grown in water culture and supplied with different CO_2 concentrations *via* the root aeration system, compared with control plants supplied with compressed air. Percentage changes were calculated from the data of tables 5A, 5B and 5C.[1]



[1], Two sets of data are presented for CO_2-free treated plants. The first bar was obtained from data in table 5a (Exp. A) and the second from data in table 5c (Exp. C) as indicated on the histogram.

Table 5A: Effect of the absence of CO_2 in the root system aeration gas on nitrogen fixation and growth of *Alnus glutinosa*.

Aeration gas	Dry matter, g.Plant^{-1}				(Root / Shoot)	Leaf area, cm^2	Nitrogen content (1), mg.		
	Shoot	Root System(2)	Nodules	Whole Plant			g^{-1} Plant dwt.	Whole plant dwt.$^{-1}$	mg^{-1} Nodule dwt.
Air	1.29 ±0.043	0.28 ±0.043	0.04 ±0.005	1.52 ±0.208	0.21 ±0.027	273.56 ±37.94	26.74 ±0.254	43.50 ±6.004	1.01 ±0.105
CO_2-free air	0.58 ±0.106	0.13 ±0.025	0.03 ±0.0004	0.72 ±0.129	0.24 ±0.039	143.00 ±24.36	23.44 ±0.935	16.87 ±2.982	0.61 ±0.115
Difference between means and its level of significance(3)	0.695	0.015	0.016	0.798	-0.030	130.56	3.29	26.66	0.393
	0.003 ***	0.012 **	0.034 **	0.005 ***	0.462 NS	0.010 **	0.027 **	0.016 **	0.011 **

Mean ± SE (n = 9, except for total nitrogen where n = 3).

(1), Total nitrogen determinations were on combined dry matter of three plants grown in the same pot. Three pots were analysed for each treatment .

(2), Root system = roots + nodules.

(3), ***, significant at $P \leq 0.01$; ** , significant at $P \leq 0.05$ and NS, not significant.

The plants were harvested after 42 days of growth.

Table 5B: Effect of elevated CO_2 content in the root system aeration gas on nitrogen fixation and growth of *Alnus glutinosa*.

Aeration gas	Dry matter, g . Plant^{-1}				(Root / Shoot)	Leaf area, cm^2	Nitrogen content (1), mg.		
	Shoot	Root System(2)	Nodules	Whole Plant			g^{-1} Plant dwt.	Whole plant dwt.$^{-1}$	mg^{-1} Nodule dwt.
Air	1.67 ±0.161	0.44 ±0.036	0.068 ±0.007	2.13 ±0.194	0.27 ±0.015	389.67 ±28.86	26.09 ±0.956	55.27 ±1.135	0.82 ±0.075
Air with 1.5% CO_2	2.36 ±0.397	0.55 ±0.090	0.072 ±0.015	2.89 ±0.488	0.24 ±0.013	489.44 ±65.57	27.74 ±0.312	80.26 ±3.071	1.14 ±0.144
Difference between means and its level of significance(3)	0.686	0.106	0.004	0.769	-0.030	99.777	1.642	24.984	0.315
	0.129 NS	0.289 NS	0.780 NS	0.162 NS	0.149 NS	0.182 NS	0.178 NS	0.001 ***	0.124 NS

Mean ± SE (n = 9, except for total nitrogen where n = 3).

(1), Total nitrogen determinations were on combined dry matter of three plants grown in the same pot. Three pots were analysed for each treatment.

(2), Root system = roots + nodules.

(3) ***, significant at $P \leq 0.01$ and NS, not significant.

The plants were harvested after 42 days of growth.

Table 5C: Effect of CO_2 levels in the root system aeration gas on nitrogen fixation and growth of *Alnus glutinosa* .

Aeration gas		Dry matter, g.Plant^{-1}				(Root / Shoot)	Leaf area, cm^2	Nitrogen content (1), mg.		
		Shoot	Root System(2)	Nodules	Whole Plant			g^{-1} Plant dwt.	Whole plant dwt.$^{-1}$	mg^{-1} Nodule dwt.
1- CO_2-free air		0.64 ±0.081	0.11 ±0.017	0.028 ±0.008	0.75 ±0.094	0.17 ±0.032	NM	22.91 ±0.324	17.21 ±0.798	0.60 ±0.062
2- Air		0.97 ±0.131	0.14 ±0.011	0.036 ±0.005	1.10 ±0.14	0.16 ±0.021	NM	24.31 ±0.774	26.87 ±2.566	0.74 ±0.037
3- Air with 5% CO_2.		0.85 ±0.085	0.11 ±0.013	0.027 ±0.004	0.96 ±0.090	0.13 ±0.028	NM	24.56 ±0.434	23.77 ±2.936	0.86 ±0.081
Differences between	(2-1)	0.322	0.029	0.007	0.357	-0.017		1.316	9.630	0.136
means and levels of significance(3)		0.053 *	0.188 NS	0.356 NS	0.05 **	0.449 NS		0.192 NS	0.023 **	0.132 NS
	(2-3)	0.119	0.033	0.008	0.141	0.024		0.248	3.074	0.123
		0.459 NS	0.085 *	0.239 NS	0.411 NS	0.241 NS		0.793 NS	0.474 NS	0.238 NS

Mean ± SE (n = 9, except for total nitrogen where n =3).

(1), Total nitrogen determination were on combined dry matter of three plants grown in the same pot. Three pots were analysed for each treatment .

(2), Root system = roots + nodules . (3), **, significant at $P \leq 0.05$; *, significant at $P \leq 0.1$; and NS, not significant.

NM, not measured.

The plants were harvested after 34 days of growth.

It can be seen from table 5A that the removal of CO_2 from the aeration stream had significant effects ($P \leq 0.05$) on all plant characteristics measured except root/shoot ratio. The effects on dry matter production of shoots and whole plants were statistically significant even at $P \leq 0.01$. When dry weights of treated plants were plotted as % of those of control (Fig. 4), it can be seen that the absence of CO_2 in the aeration stream decreased the yield (dry matter) of the shoot, root system (roots + nodules) and of the whole plant by around 50% in each case.

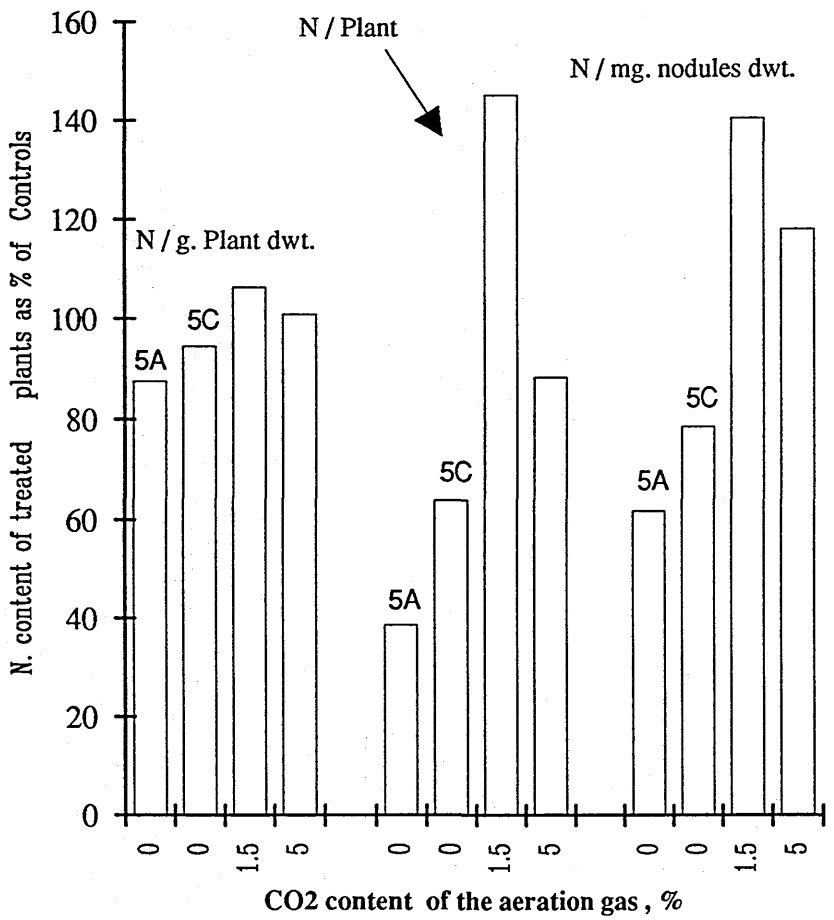
Table 5B shows that the apparent increases in shoot, root system and nodule dry matter of plants subjected to an aeration stream containing 1.5% CO_2, compared to those of atmospheric air-treated plants (Fig. 4), were not statistically significant.

In experiment C, Table 5C shows that the removal of CO_2 from the aeration stream caused measurable decreases (see differences betwwen means) in dry matter of plant parameters measured, except for root/shoot ratio. However, it can be seen from Figure 4 that these decreases were not as big as those recorded in experiment A. Statistical analyses (Table 5C) show that the only statistically significant effects were on whole plant ($P \leq 0.05$) and shoot ($P \leq 0.10$) dry weights, which were decreased by 25% and 35%, respectively (Fig. 4). This difference could be due to this experiment being run ten days fewer than experiment A as the gas supply ran out prematurely. Table 5C also shows that the apparent decreases in dry matter of all parts of the plants aerated with air containing 5% CO_2, compared to controls (Fig. 4) were not statistically significant, with the exception of that of root system dry matter which was statistically significant at $P \leq 0.10$.

2.3.2.3. Comparison of nitrogen content.

Data for nitrogen contents are shown in tables 5A, 5B and 5C while comparisons between the nitrogen content of treated plants and that of controls in each experiment are summarised in figure 5.

Figure 5. Nitrogen content of *Alnus glutinosa* plants grown in water culture and supplied with different CO_2 concentrations *via* the root aeration system, compared with control plants supplied with compressed air. Percentage changes were calculated from the data of tables 5A, 5B and 5C.[1]



[1], Two sets of data are presented for CO_2-free treated plants. The first bar was obtained from data in table 5a and the second from data in table 5c as indicated on the histogram.

It can be seen from figure 5 that omission of CO_2 from the aeration gas (Exp. A) decreased the specific nitrogen content by about 13%, the nodule specific

activity by 40% and total nitrogen per plant by 60%, compared to controls. These decreases were statistically significant at $P \leq 0.05$ and correlated with inhibitory effects on the dry matter production (Table 5A).

Table 5B shows that the apparent increases in the specific N content and the nodule specific activity (Fig. 5) that resulted from elevation of aeration gas CO_2 content to 1.5% were not statistically significant, whereas an increase of 45% in total nitrogen per plant (Fig. 5) was highly significant ($P \leq 0.01$).

In experiment C, the effects of the absence of CO_2 from the aeration stream on nitrogen fixation were less than those recorded in experiment A. Table 5C shows that only a reduction in total nitrogen per plant of 36% (Fig. 5), compared with controls, achieved statistical significance ($P \leq 0.05$).

Inclusion of 5% CO_2 in the aeration stream resulted in less nodule specific activity and smaller content of nitrogen per plant (Fig. 5), compared to controls, but none of these differences were statistically significant (Table 5C).

2.4. DISCUSSION.

The aim of this study was to investigate the effects of both short-term and long-term changes in CO_2 concentrations around the root systems on nitrogen fixation and growth of the alder plant *Alnus glutinosa.*

2.4.1. Effects of short-term changes.

In short-term experiments, effects on nitrogenase activity (nitrogen fixation) were determined using the acetylene reduction assay. Root systems of intact plants were used in this assay because previous studies showed that they have an acetylene reduction rate 2 and 5 times higher than that of decapitated root systems and of detached nodules, respectively (Mague & Burris, 1972). Other advantages of using root systems of intact plants in acetylene reduction assays include: (a) retention of functional xylem and phloem transport systems during the experiment, (b) the possibility of monitoring other physiological processes, such as CO_2-exchange and transpiration, simultaneously with nitrogenase activity measurements and (c) the ability to measure repeatedly the nitrogenase activity of the same plant so that the number of plants required for an experiment can be reduced (Huss-Danell, 1978).

As already mentioned, since its first description in the 1960's the acetylene reduction method has been used extensively as a sensitive assay for nitrogen-fixation and for studying the relationship of this process with dark CO_2 fixation in different plant materials (eg., detached nodules, decapitated intact root systems or the root systems of intact plants). However, recent work with both legumes and actinorhizal plants has emphasised the need to treat the quantitative relationship between N_2-fixation and C_2H_2 reduction with caution.

Of particular note is the data of Minchin *et al.*, (1983) who were the first to report what is known now as the acetylene-induced decline phenomenon. These researchers noted that when nodulated white clover roots of intact plants were

exposed to acetylene (10%) in a closed system, the ethylene evolution (nitrogenase activity) increased during an initial 4 min (attributed to the time required for acetylene equiliberation in the assay vessel), stabilized for about 2 min, and then declined rapidly to a new steady state within about 10 min. In a second experiment in which detached nodules were exposed first to $^{15}N_2$ and then to C_2H_2, they found that the measured ratios of acetylene reduction to N_2-fixation ($^{15}N_2$-uptake) were close to calculated values when estimated from maximum (pre-decline) rates of ethylene evolution. From this, the authors concluded that it is the maximum, rather than the mean or final, rate of acetylene reduction which most nearly represents the pre-assay rate of nitrogenase activity. Therefore, calculations of nitrogenase rates based on cumulative ethylene production, as is the case in most of published work, will underestimate actual rates and produce low apparent values for acetylene reduction that are lower than the true rates of reduction. The error will, of course, be proportional to the magnitude of the decline. Not all legumes tested show this phenomenon, for reasons that are still unclear (Minchin & Witty, 1989).

Actinorhizal nodules also show a range of responses to acetylene exposure. In *Datisca* and *Myrica* there is a decline followed by a limited (40 to 60%) recovery (Monz & Schwintzer, 1989). In *Alnus*, the genus used in the present study, however, the decline is followed by a recovery to rates about 80 to 85% of the initial maximum rate (Tjepkema, 1989; Silvester & Winship, 1990).

The use of the acetylene reduction technique in this study can be further justified by the comparative nature of the experiments, in which treated plants are always compared with controls.

In the present short-term acetylene reduction experiments, the ethylene evolution rates were apparently linear for 6 hours when CO_2 was not added to the assay jars (Fig. 2, graph A), but it should be noted that the initial changes in ethylene evolution rates were not determined. The first samples for determination of ethylene evolution rates were taken 1 hr after addition of C_2H_2. The scale of a

possible ethylene-induced decline thus has not been determined.

It should also be noted that in these experiments, evolution of respiratory CO_2 by the root systems will have increased the CO_2 content of the closed incubation jars by 0.5% after 3 hours and 1% after 6 hours (Tab. 4). These increases should be taken into consideration when the effects of changes in [CO_2] around the root systems on acetylene reduction are interpreted.

The effects of elevated [CO_2] on acetylene reduction noted in Fig. 2 (graphs, B - F), may be interpreted in relation to the well documented relationship between supply of additional carbon through dark carboxylation reactions and nitrogenase activity of nodulated legumes and alders (see introduction). The range of added CO_2 concentrations (1.1 to 1.75%) found to stimulate acetylene reduction (graphs C & D), plus up to 1% CO_2 arising from root respiration falls within the limits of the 1 to 3% CO_2 reported to saturate the dark CO_2-fixing processes in soybean attached nodules (Coker & Schubert, 1981); although, Vance *et al.* (1983) reported that CO_2 concentrations between 4.9 and 8.4% were required to saturate dark CO_2-fixation in excised alfafa nodules. In alders, McClure *et al.* (1983) obtained elevated rates of dark CO_2 fixation for detached nodules of *A. glutinosa* at [CO_2] between 1.1 and 1.7%, but these researchers did not determine the [CO_2] required to saturate the system. Furthermore, these stimulatory effects (graphs C & D) are in agreement with the results of Bethenod *et al.* (1984) who reported a 40% increase in acetylene reduction activity when the CO_2 levels around the nodulated roots of various leguminous plants were increased from less than 0.01% to 0.2 - 0.3%. Both sets of data may be attributable to an increase in available organic acids, major products of dark CO_2 fixation, the oxidation of which will increase the nitrogenase activity by energy provision. For instance, King *et al.* (1986) have concluded from their study of soybean that at least 66% of the products of dark CO_2 fixation are involved in the biosynthesis of organic acids, which when oxidized could provide at least 48% of the requirements for ATP to support nitrogenase activity.

The inhibitory effects on acetylene reduction obtained when 2.5% or 4.85% CO_2 were added to the jar gas-phase (graphs, E & F) may be related to published data on the inhibitory effects of CO_2 on PEPc, the main enzyme in the dark CO_2-fixation process. Christeller *et al.* (1977) have reported that at both saturating and sub-saturating [PEP] the K_m (CO_2) values calculated for partly purified PEPc from lupin root nodules were less than the equivalent of 0.01% CO_2 in the gas-phase over the reaction mixture. From this, they postulated that PEPc in nodules should be saturated by CO_2 even in air levels (0.03%). These researchers did not measure the [CO_2] required to inhibit PEPc. However, an early study by Walker & Brown (1957) showed that the level of CO_2 required to saturate the activity of PEPc, extracted from *Kalanchoe* leaves, was in the region of 0.5%. As [CO_2] increased further, enzyme activity was not much affected until a level of 3% was attained, after which there was some inhibition, but the enzyme was still considerably active even in the presence of 10% CO_2.

2.4.2. Effects of long-term changes.

The substantial effects on acetylene reduction of increasing the CO_2 content of the rooting atmosphere, suggested from the short-term acetylene reduction experiments, did not translate readily into similar statistically significant effects on plant biomass production and nitrogen fixation in the long-term experiments. One explanation for this result could be that plant-to-plant variation masked any changes which did occur. Thus, both the apparent 35% increase in whole plant dry weight and the 40% increase in nodule specific activity of plants aerated with air containing 1.5% CO_2, compared with controls, failed to reach statistical significance (Table 5b). Alternatively, since visual inspections of the plants showed that these growth differences were greater at earlier stages in the experiment but started to disappear towards the end of the experiment, it is conceivable that there could be some adaptation to different root atmospheres during plant growth. On the basis of this

explanation, the apparent decrease in biomass of plants receiving 5% CO_2 in the aeration stream might also have been relatively larger early in the experiment compared with those differences which were obtained at harvest (Table 5c). However, as already mentioned in the introduction, published data provide conflicting information concerning the levels of root CO_2 required for inhibition of nitrogen fixation and plant growth (see Mulder & Van Veen, 1960; Grobbellar *et al.*, 1971).

Enrichment of the root system atmosphere with 1.5% CO_2 resulted in a highly significant ($P \leq 0.01$) increase in total nitrogen per plant, compared with compressed air-aerated controls. Such difference could be the result of changes either in nodule biomass per plant or in nodule specific activity. However, the apparent increases in values for the latter two parameters compared to the respective values for air controls (Figures, 4 & 5) failed to achieve statistical significance (Table 5b), due to plant-to-plant variation. From the differences in mean values and their levels of significance (Table 5b), it appears that a major part of the significant increase in total nitrogen per plant could be the result of a relatively large increase in nodule specific activity (nitrogenase activity) alone since nodule biomass per plant is less affected. However, the possibility that another factor, not measured here, is the main cause of this increase can not be ruled out.

The significant inhibition of dry matter accumulation and N_2-fixation that resulted from removal of all CO_2 from the air supply to the jars in experiment (A) suggests that root respiration may be unable to generate sufficient CO_2 to maintain a flux of HCO_3^- sufficient to support maximum growth. Thus, inhibitory effects on the biomass of the root system (Table 5a) may be attributable to a decrease in the rate of dark CO_2 fixation, and a consequent reduction in accumulation of its products in both the roots and the nodules (Coker & Schubert, 1981; McClure *et al.* 1983; King *et al.* 1986; Rosendahl *et al.* 1990). Inhibition of N_2-fixation and/or nitrogen assimilation also is a possible major cause of growth inhibition here since Bethenod *et al.* (1984) found significant inhibition of C_2H_2 reducing activity when

the CO_2 supply to the roots of a number of legumes was reduced to less than 100 vpm. According to these authors, decreased CO_2 fixation due to the lowering of CO_2 in the root atmosphere would decrease available malate and reduce nitrogenase activity by energy deprivation. Decreased nitrogenase activity and a decrease in carbon skeletons for the synthesis of nitrogenous compounds that are available for transport from the root system to the shoot could be the cause of the decrease in shoot biomass noted in Table 5a.

Comparison of the results of experiments A and C (Tables 5a and 5c, respectively) demonstrate clearly that the effects of the removal of CO_2 on plant development is cumulative and depends largely on the duration of the treatment since the effects recorded in experiment A were greater than those in experiment C which was run for a period 10 days shorter.

During the growth experiments, care was taken to pipe away from the shoots the effluent gas from the plant jars so that it is unlikely that the results obtained in the current study were influenced by enhanced photosynthesis due to elevation of the CO_2 content of the shoot atmosphere. However, the possibility that there may have been some movement of CO_2 from root to shoot within the plant cannot be ruled out. Furthermore, the question of the relationship between the effects of changes in root CO_2 supply and root medium pH has not been examined here. This will undoubtedly, as already discussed in the introduction, have substantial effects on the levels of HCO_3^- dissolved in the water. It should be noted that in a long-term experiment carried out previously with alders in culture solution at pH 4.2 (cf. pH 6.3 maintained in the present experiments), no effect on plant growth of removal of CO_2 from the aeration stream was detected (Wheeler, unpublished data). This could be due to the fact that at pH 4.2, HCO_3^-, the species for PEPc, represent only about 1% of the dissolved CO_2, whereas at pH 6.3 the percentage of HCO_3^- is around 50%. Therefore, at pH 4.2 the effects of CO_2 concentrations in the root atmosphere on carboxylation reactions are primarily dependent on the inward diffusion of CO_2

across the cellular membranes, compared to pH 6.3.

In practical terms, the results suggest that growth of alders in many ecosystems with high water tables may be sub-optimal due to the build-up of high CO_2 concentrations in the soil atmosphere. Bown (1985) reported that flooded soils CO_2 levels can rise to 12% and the extracellular levels of CO_2 in flooded tissues can exceed 5%.

CHAPTER THREE:

AUTOTROPHIC GROWTH OF *FRANKIA*.

3.1. INTRODUCTION

The symbiosis of N_2-fixing organisms with higher plants is based on exchange of nutrients between the two partners. It is well established that the host plant obtains the nitrogen fixed by the microsymbiont, mainly in the form of amides or ureides. An important question concerning this symbiosis, however, is the nature of the carbon compounds received by the microsymbiont from the host plant in the first place, metabolism of which support N_2-fixation and ancillary processes. This question can be studied in two ways: (a) by elucidation of the pathways for carbon metabolism of the bacteria when grown in culture - study of which will give an overall view of the potential for metabolism of C-compounds, and (b) by investigation of C uptake and metabolism by the microsymbiont "*in vivo*" - technically more difficult but providing a direct insight into the metabolic pathways employed during symbiosis.

In *Rhizobium* both approaches have been used to investigate C metabolism. In *Frankia*, however, studies of "*in vivo*" metabolism are technically more difficult because of problems of separation of the intracellular mycelium of the microsymbiont from the host cytoplasm. Consequently, most of the data available to date have been obtained from "*in vitro*" studies.

3.1.1. Heterotrophic growth.

3.1.1.1. Rhizobia.

3.1.1.1.1 *In vitro* state (in culture).

Numerous investigations have examined the diversity of carbon compounds utilized by rhizobia "*in vitro*" and have shown that compounds respired include, hexoses, pentoses, trisaccharides, disaccharides, amino acids and organic acids from the TCA (reviewed in Stowers, 1985_b; O'Gara *et al.*, 1989); glycerol is the most universally used carbon source among rhizobia (Arias & Martinez-Drets, 1976). From data

obtained from these investigations rhizobia can be divided physiologically into two broad groups differing in their generation times, ability to utilize particular compounds, especially disaccharides, and the type of reaction they produce when grown on yeast-manitol or other carbohydrates (Jordan, 1984; Padmanabhan *et al.*, 1990): (a) fast-growing rhizobia with generation times of less then 4-6 hours, such as *R. leguminosarum*, *R. meliloti*, *R. phaseoli* and *R. trifoli*, are able to use a broad range of hexoses, pentoses, disaccharides, trisaccharides, and organic acids, and produce an acidic reaction in mineral-salts media containing manitol or other carbohydrates, and (b) slow-growing rhizobia (Bradyrhizobium) with generation times exceeding 6 hours, such as *B. japonicum*, *B. lupini* and cowpea miscellany rhizobia, are more limited in their ability to use diverse carbon sources. For instance, they are unable to use disaccharides and prefer pentoses as carbon sources. These strains produce an alkaline reaction in mineral-salts media containing manitol or other carbohydrates.

Free-living rhizobia have several pathways available for carbon metabolism. The Entner-Doudoroff (ED) pathway, the Embden-Meyerhof-Parnas (EMP) pathway, the tricarboxylic acid (TCA) cycle and various anapleurotic sequences are all functional in most rhizobia examined. The pentose phosphate (PP) pathway, however, is restricted to fast-growing strains only (reviewed in Stowers$_b$, 1985).

3.1.1.1.2. Symbiotic state (*in planta*).

In the symbiotic state, a variety of potential carbon and energy sources for rhizobia bacteroids are available in the host-cell cytosol. Sucrose, the major photosynthetic product transported from the shoots *via* the phloem to the nodules (Pate, 1975; Kouchi & Yoneyama, 1984), glucose, fructose, aldehydes and alcohols have all been found in nodules of leguminous plants (Bach *et al.*, 1958; Robertson & Taylor, 1973; Lawrie & Wheeler, 1975; Kouchi & Nagaji, 1985).

Available data, however, suggest that the utilization of sucrose or the

hexoses produced by its hydrolysis as primary energy sources for N_2-fixation by bacteroids is unlikely. For example, although enzymes for hexose catabolism have been reported in *R. leguminosarum* bacteroids, mutants lacking these enzymes can form effective nitrogen-fixing nodules (Arias *et al.* , 1979; Glenn *et al.*, 1984). Furthermore, studies by Reibach & Streeter (1983) and Salminen & Streeter (1987) showed that the glucose and fructose that are taken up are metabolised slowly because *B. japonicum* bacteroids lack, or have very low levels of, phosphofructokinase (E.C. 2.7.1.11) and NADP-dependent 6-phosphogluconate dehydrogenase (E.C. 1.1.1.4); key enzymes in the EMP and PP pathways, respectively. These findings are in agreement with earlier investigations which showed that sugars are generally not metabolized by *Rhizobium* bacteroids (e.g., Bergersen & Turner, 1975).

Recent studies have shown that the primary energy sources for bacteroids are organic acids, aldehydes and alcohols, not hexoses (reviewed in O'Gara *et al.*, 1989; McDermott, 1989). Dicarboxylic acids found in the soybean nodule cytosol (Streeter, 1987), are actively transported (San Francisco & Jacobson, 1985; Kouchi *et al.*, 1988) at rates 30 to 50-fold those of sugars (Salminen & Streeter, 1987) and are the most effective substrates for promoting N_2-fixation by isolated bacteroids (Peterson & LaRue, 1982; Kouchi & Nagaji, 1985; Bolton *et al.*, 1986). The isolation of mutants of a number of rhizobia strains defective in the dicarboxylic acid transport (Dct) system has also indicated the vital role played by these compounds in nodule metabolism and nitrogen fixation. Mutants lacking C_4 dicarboxylate transport systems or a complete TCA cycle form nodules that are ineffective in fixing nitrogen (reviewed in $Stowers_b$, 1985).

Compounds such as poly-β-hydroxy-butyrate (PHB) and starch are stored in large amounts in rhizobial nodules (e.g., Wong & Evans, 1971; Minchin & Pate; 1974; Gerson *et al.*, 1978). However, although Wong & Evans (1971) and Werner & Morschel (1978) showed that PHB could represent up to 50% of cell dry weight,

its role in nodule and bacteroid metabolism is still not clear. It has been suggested that it may play an important role under conditions of limited photosynthate supply or during the dark periods (Minchin & Pate, 1974; Peterson & LaRue, 1981).

In addition to photosynthates and stored compounds, the anapleurotic reactions occurring in the nodules also play an important role in the carbon economy and nitrogen fixation of the rhizobia-legumes symbiotic relationship. Many data supporting the idea that dark CO_2-fixation catalysed by nodule PEPc may provide an additional source of carbon for nodule development, maintenance and assimilation of fixed nitrogen have been obtained in the last 15 years (e.g., Lawrie & Wheeler, 1975; Christeller *et al.*, 1977; Vance *et al.*, 1985; King *et al.*, 1986; Ta *et al.*, 1987). Nodule CO_2 fixation was estimated to provide 25% of the carbon required for assimilation of symbiotically fixed nitrogen in alfafa (Vance *et al.*, 1983). Furthermore, King *et al.* (1986) estimated that oxidation of organic acids, products of dark CO_2 fixation in soybean root nodules, could provide at least 48% of the energy required for nitrogenase acitvity. In a more recent study, Rosendahl *et al.* (1990) compared the distribution of the metabolites of nodule dark CO_2 fixation in bacteroids of nodules infected by an effective wild type *R. leguminosarum* (MNF 300), and nodules infected by the *R. leguminosarum* mutant MNF 3080 which has a defect in the dicarboxylic acid transport (Dct) system. They found that: (a) the amount of ^{14}C accumulated in the bacteroids from nodules infected by the wild type was 7 times greater than that in the dicarboxylic acid transport defective bacteroids, (b) the bacteroids of MNF 300 contained the largest proportion of ^{14}C in the organic acids, whereas bacteroids of MNF 3080 mainly contained ^{14}C in the amino acid fraction, and (c) the proportion of ^{14}C in succinate, 2-oxoglutarate, citrate and fumarate in the bacteroids of the wild type greatly exceeded that of the Dct-mutant. These data provided conclusive evidence that products of dark CO_2 fixation, which occurs in the plant cytosol of pea root nodules, are taken up by the bacteroids.

3.1.1.2. *Frankia.*

Unlike rhizobia, *Frankia* is a rather slow-growing organism with generation times between 2 and 5 days (Diem & Dommergues, 1983). Therefore, determination of the ability to grow on a given substrate can be a lengthy process, which may involve growth for weeks in the appropriate medium before the ability to utilize the substrate of interest is proved or rejected.

3.1.1.2.1. *In vitro* state.

In vitro nutritional studies have shown that a wide variety of carbon compounds can serve as the sole carbon source for growth of free-living *Frankia*, although marked differences in patterns of carbon utilization exist between different strains.

The most effective carbon sources include short-chain fatty acids like propionate and acetate, fatty acid derivatives such as Tweens, TCA cycle intermediates like succinate or malate, other organic acids like pyruvate, and in some cases sugars (Blom, 1982; Shipton & Burggraaf, 1982; Akkermans *et al.*, 1983; Burggraaf & Shipton, 1983; Shipton & Burggraaf, 1983; Tisa *et al.*, 1983; Zhang *et al.*, 1986; Lopez *et al.*, 1986). Not all strains use all of these compounds, although some strains can adapt to grow on substrates they do not usually use. For example, Shipton & Burggraaf (1983) did not detect growth of Cas JCT287, a *Frankia* isolate from *Casuarina equisetifolia*, on glucose after 20 days of incubation, but Burggraaf (cited in Shipton & Burggraaf, 1983) showed utilization of glucose by the same strain following a longer period of incubation. Growth on these substrates, however, is only rarely superior to growth on organic acids like propionate (Burggraaf & Shipton, 1983; Tisa *et al.*, 1983; Lechevalier & Ruan, 1984). Both age and pregrowth conditions of inoculum may influence the speed of adaptation to substrates (Shipton & Burggraaf, 1983; Murry *et al.*, 1984).

The inability of some *Frankia* strains to use carbohydrates, amino acids or organic dicarboxylic acids has been attributed to the lack of a proper uptake system

for these compounds or to the lack of enzymes needed for the degradation of these compounds (Akkermans *et al.*, 1983; Lechevalier & Ruan, 1984). For example, glyoxylate-cycle enzymes like isocitrate lyase (ICL) and malate synthase (MS) are usually repressed in microorganisms when succinate or succinate precursors are available in the growth medium, as shown for *Frankia* AvcI1 growing in media with propionate as C-source (Blom, 1982). No repression occurred, however, when the same organism was cultivated in media with acetate or succinate as C-source, which was possibly due to the inability to take up succinate in sufficiently high quantities (Akkermans *et al.*, 1983).

Some compounds may also be utilized only when additional carbon sources are present in the growth medium. For instance, addition of Tweens to growth media usually improve the growth response on other C substrates such as amino acids (Blom *et al.*, 1980; Blom, 1982) and carbohydrates (Lechevalier & Ruan, 1984). Tweens presumably act by increasing the permeability of cell membranes.

Physiologically, *Frankia* strains have been divided into two groups which differ, among other aspects, in their ability to utilize carbohydrates as carbon source (Lechevalier & Lechevalier, 1989). Group A strains, generally isolated from *Elaeagnus* (Benson & Schultz, 1990), are heterogenous, aerobic and able to grow on carbohydrates such as glucose, sucrose, maltose and trehalose. Group B strains, generally isolated from *Alnus*, *Comptonia* or *Myrica* (Benson & Schultz, 1990), are more homogenous, less tolerant to O_2 and prefer short-chain fatty acids or TCA intermediates, although some of these also use carbohydrates as carbon source for their growth (Shipton & Burggraaf, 1982; Lopez & Torrey, 1985).

Because the main storage compounds in cultured *Frankia*, even in strains that grow poorly on carbohydrates, are glycogen or trehalose (Benson & Eveleigh, 1979; Lopez *et al.*,1984) most, if not all, strains probably posses enzymes for carbohydrate catabolism. However, data obtained from studies on the metabolic pathways employed by free-living *Frankia*, most of which was carried out with

strains from group B, are conflicting with regard to the metabolic pathways used for catabolizing carbohydrates. Early investigations by Blom & Harkink (1981), for example, failed to detect enzymes of the Emden-Meyerhof-Parnas (EMP) pathway such as hexokinase (HK), pyruvate kinase (PK) and pyruvate dehydrogenase (PDH) in *Frankia* sp. AvcI1 growing on Tween medium. Whereas, recent studies demonstrated the presence of key enzymes of the EMP, such as glucokinase, phosphofructokinase and pyruvate kinase in cell-extracts of *Frankia* sp. HFPArI3 growing on propionate and trehalose (Lopez & Torrey, 1985) and *Frankia* sp. NPI 0136010 growing on propionate (Stowers *et al.* , 1986).

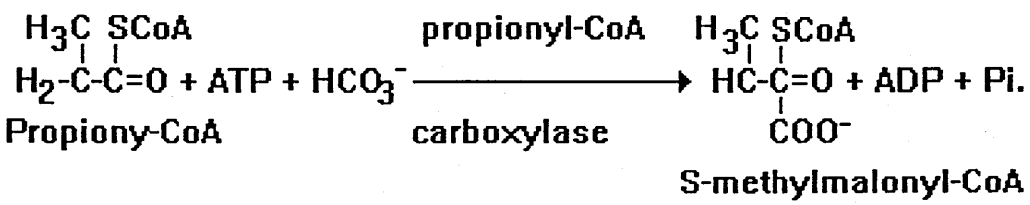
Enzymes of the PP pathway such as glucose-6-phosphate dehydrogenase (G-6-DH) and 6-phosphogluconate dehydrogenase (6-PGDH) were also detected in cell-extracts of HFPArI3 growing on propionate (Lopez & Torrey, 1985) but were not detected in cell-extracts of NPI 0136010 growing on propionate too (Stowers *et al.* ,1986). Both investigations (Lopez & Torrey, 1985; Stowers *et al.* ,1986) failed to detect enzymes of the ED pathway, but this is not surprising since this pathway usually functions in gram-negative bacteria such as *Pseudomonas* (Lessie & Phibbs, 1984) and *Rhizobium* (Stowers & Elkan, 1983; Stowers_b, 1985). The data presented by Lopez & Torrey (1985) suggests that in *Frankia*, a gram-positive organism, glucose is catabolised through the Embden-Meyerhof-Parnas pathway, as in other actinomycetes (Cochrane, 1955).

Tricarboxylic acid cycle, glyoxylate cycle, and gluconeogenic enzyme activities have been demonstrated in *Frankia* AvcI1 when growing under the appropriate conditions (Blom & Harkink, 1981; Blom, 1982; Akkermans *et al.*, 1983; Lopez & Torrey, 1985; Stowers *et al.*, 1986). The detection of these enzymes is not surprising since most *Frankia* tested so far grow well on propionate, acetate and in some cases succinate.

Propionate is the most widely utilized carbon source for *Frankia* growth, since all, but one (Tisa *et al.*, 1983), *Frankia* strains tested so far grow to some

degree on media containing propionate as sole carbon source. Propionate is probably metabolized to succinate *via* methyl malonyl CoA in a reaction catalysed by the vitamin B12 requiring enzyme, methyl malonyl mutase (Blom & Harkink, 1981; Stowers *et al.*, 1986). This is the most frequently employed pathway for propionate catabolism in bacteria (Wegener *et al.*, 1968).

In this pathway the carboxylation enzyme propionyl-CoA carboxylase acts on propionate *via* propionyl CoA, which has been found in *Frankia* cell-free extracts (Blom, unpublished data, cited in Akkermans *et al.*, 1983), using HCO_3^- / CO_2 to produce S-methyl-malonyl CoA.



This reaction can progress further by isomerisation of the product by the methylmalonyl-CoA racemase and methylmalonyl-CoA mutase enzymes, respectively, to form the dicarboxylic acid, succinate *via* succinyl CoA. The repressing effect of propionate on the glyoxylate cycle enzymes in cell-extracts of AvcI1 (Blom, 1982) supports the idea that propionate is metabolized *via* succinate as found in *E. coli* (Wegener *et al.*, 1968). The resulting succinate can be further metabolized to pyruvate *via* β-oxidation and decarboxylation (Stowers *et al.*, 1986).

Stimulation of growth of ArI3 (Murry *et al.*, 1984) and a number of other *Frankia* strains (Shipton & Burggraaf, 1982) on propionic acid following the addition of biotin is another strong indication that this pathway is operative at least in some strains since carboxylation of propionyl CoA to methyl-malonyl CoA is biotin-dependent. However, the failure of some strains to respond to biotin addition when grown on propionate (Shipton & Burggraaf, 1982) may indicate either a synthetic ability for this vitamin or alternative pathways for the breakdown of propionate. Therefore, it seems that not all free-living *Frankia* strains are alike in

their metabolic pathways.

3.1.1.2.2. Symbiotic state.

As in symbiotic rhizobia, a good supply of carbon is essential for maintenance, growth processes and nitrogen fixation in symbiotic *Frankia.* Wheeler & Lawrie (1976) detected some radioactivity, although small, in infected cells just 5 hours after supplying $^{14}CO_2$ to the shoots of *A. glutinosa* seedlings. Photosynthates are rapidly translocated to and metabolized on arrival in nodules but various problems related to the nature of *Frankia "in vivo"* have made resolution of the nature of carbon compounds transported from the host to symbiotic *Frankia* difficult.

Carbon metabolism in symbiotic *Frankia* has been studied using vesicle cluster suspensions derived from infected cells of root nodules. "Vesicle" is a term applied to terminal swelling on the tips of frankial hyphae in symbiosis or in cultures starved of nitrogen. A major problem with experiments carried out with these vesicle clusters is the difficulty of separating plant cytoplasm from the *Frankia* mycelium so that metabolism can not be ascribed unequivocally to the host plant or the bacterium. Therefore, it is not surprising that early data obtained from studies on these preparations were rather conflicting. For instance, Huss-Danell *et al.* (1982) failed to detect activities for the EMP pathway enzymes in vesicle clusters prepared from nodules formed on *A. glutinosa* roots by *Frankia* AvcI1, whereas Lopez & Torrey (1985) detected activities of enzymes catalysing irreversible steps in the EMP in vesicle clusters from nodules containing *Frankia* ArI3. Moreover, succinate was shown earlier to stimulate respiration by vesicle clusters obtained from *A. glutinosa* nodules formed after infection with a nodule homogenate (Akkermans *et al.*, 1981), whereas Lopez *et al.* (1986) showed that vesicle clusters isolated from nodules formed after inoculation of *A. rubra* with ArI3 would not respire some organic acids, including succinate. The loss of the uptake system for organic acids during the problematic vesicle cluster isolation

process, however, could not be ruled out.

Enzymes of the TCA cycle are active in vesicle clusters (Akkermans *et al.*, 1981). Lipids, however, are not a likely carbon source for symbiotic *Frankia*, since Huss-Danell *et al.* (1982) failed to detect activities of the glyoxylate cycle enzymes isocitrate lyase and malate synthetase in vesicle clusters prepared from nodules containing *Frankia* AvcI1, although Blom & Harkink (1981) detected activities for these enzymes in extracts of the same *Frankia* strain growing in a medium containing the lipid compound Tween 80.

In 1987, Vikman & Huss-Danell (1987_b) obtained vesicle clusters with high purity such that *Frankia* vesicle clusters constituted at least 98% of the particle volume in the preparations. Studies with these preparations (Vikman & Huss-Dunell, 1987_a) helped clarify the nature of the hexose-respiratory pathways operative in symbiotic *Frankia*. They showed that the phosphorylated sugars glucose-6-phosphate and 6-phosphogluconate both stimulated respiration, provided that cofactors were added to the preparations. The hexose-degrading enzymes, HK, G-6-PDH, and 6-PGDH were detected in all *Frankia* cells investigated, even though the cells were not supplied with hexoses. This suggested that the starting compound for the employed hexose-degradation pathway does not necessarily come from the host plant, but it might be a storage compound synthesized inside the *Frankia* cells - glycogen and trehalose have both been extracted from free-living *Frankia* ArI3 (Lopez *et al.*, 1984). The cofactor-dependency of 6-PGDH in the host-cell extracts ($NADP^+$) was different from that in *Frankia* cell-free extracts (NAD^+). This provided strong evidence that the detected activity of 6-PGDH, and probably all other enzymes in cell-free extracts of symbiotic *Frankia* investigated in this study, was not a result of host-cell contamination.

Although the occurrence of CO_2 dark fixation in nodules of actinorhizas is well established, the role of this phenomena in nodule metabolism is still not clear. Labelling studies by McClure *et al.* (1983) demonstrated the existence of two pools

of malate, the major product of nodule CO_2 fixation, in nodules of *A. glutinosa*. The small pool was rapidly converted to citrate and subsequently used to make glutamate while the major pool of malate was metabolically inactive.

3.1.2. Autotrophic growth.

An autotroph has been defined as an organism that is capable of utilizing CO_2 as its major source of carbon (Woods & Lascelles, 1954; Quayle & Ferenci, 1978). According to the latter authors the inability to synthesize a vitamin would not exclude an organism from being an autotroph. Several studies (eg., Buchanan & Gibbons, 1974; Maier *et al.*, 1978; Hanus *et al.*, 1979; Malik & Schlegel, 1981) have shown that in the absence of an organic carbon substrate a wide range of these autotrophic bacteria, including some N_2-fixing species, can use H_2 to provide energy and reducing power for growth and CO_2 fixation.

3.1.2.1. Rhizobia

3.1.2.1.1. H_2-oxidation by legume root nodules.

Phelps & Wilson (1941) were the first to report the presence of an uptake hydrogenase in root nodules of *Pisum sativum* formed by *R. leguminosarum* strain 311. This discovery was not confirmed until the late 1960's when Dixon (1967, 1968) showed that no hydrogen was evolved from pea root nodules. This author was also able to establish the existence of two separate hydrogenase systems, one system evolved H_2 *via* nitrogenase and the other took up H_2 using a hydrogenase which was O_2-dependent. Later work (see Burns & Hardy, 1975) showed that H_2 is evolved from nitrogenase during nitrogen reduction and that, in the absence of any other reducible substrates (e.g., if N_2 in air is replaced by argon), the rate of hydrogen evolution should represent the total electron flux to the nitrogenase system. Furthermore, when saturating concentrations of C_2H_2 are used H_2 formation is competely inhibited and consequently, rates of acetylene reduction may be used to

estimate the total electron flux through the nitrogenase system. Based on these findings Schubert & Evans (1976) speculated that the rate of nitrogen reduction in intact nodules should be equivalent to the difference between the rate of H_2 evolution in a mixture of Argon, O_2, and CO_2 or the rate of C_2H_2 evolution and the rate of H_2 evolution in air. These authors defined the relative efficiency of electron transfer to nitrogen *via* nitrogenase by the following formula:

$$\text{Relative efficiency} = 1 - \frac{\text{rate of } H_2 \text{ evolution in air}}{\begin{matrix}\text{rate of } H_2 \text{ evolution in Ar : } O_2 : CO_2 \\ \text{or} \\ \text{rate of } C_2H_2 \text{ evolution.}\end{matrix}}$$

3.1.2.1.2. The role of the host plant.

Using this formula, Carter *et al.* (1978) surveyed hydrogen evolution by two soybean cultivars inoculated with several strains of *R. japonicum*. It was found that all nodules that failed to exhibit H_2 evolution in air took up H_2 when this gas was provided in the mixture over the cultures, indicating the presence of an uptake hydrogenase in these nodules. The remaining nodules evolved H_2 and had low relative efficiencies, based on C_2H_2 reduction, ranging from 0.41 to 0.80, compared to 0.96 to 1.0 for non-H_2-evolving nodules. In a second experiment, two strains, one H_2-evolving (H_2 uptake negative, Hup^-) and the other non-H_2-evolving (H_2 uptake positive, HuP^+), were tested on seven different soybean cultivars to determine the effect of the host plant on the expression of hydrogenase. The results showed that nodules formed by the non-H_2-evolving strain exhibited an efficiency of 1.0 whereas nodules formed by the H_2-evolving strain exhibited relative efficiencies ranging from 0.63 to 0.77. These results suggested that the capacity to recycle H_2 produced *via* nitrogenase in symbiotic associations is determined by the microsymbiont.

Hydrogen is not only oxidized by the nodule bacteroid formed by Hup^+ strains of *B. japonicum* but is also utilized by free-living Hup^+ strains of *B.*

japonicum provided that appropriate conditions for expression of uptake hydrogenase are maintained in the growth medium (Lim, 1978; Maier *et al.*, 1978). For instance, Maier *et al.* (1978) demonstrated that the expression of uptake hydrogenase activity in *B. japonicum* 122 DES required low concentration of carbon substrates in the growth media, a limited partial pressure of O_2 (1-2%) and H_2 in the atmosphere over the cultures. Both sets of data supported the conclusion of Carter *et al.* (1978) and were interpreted as conclusive evidence that the genetic information for uptake hydrogenase synthesis resides in the bacteria rather than in its host. Other investigations (Bedmar *et al.*, 1983; Lopez *et al.*, 1983), however, have shown that while the possession of uptake hydrogenase is a property of the microsymbiont, the host plant can exert a modifying influence.

3.1.2.1.3. Benefits from H_2-oxidation.

Symbiotic state:

Evans *et al.* (1983) found that plants inoculated with the Hup^+ *R. japonicum* strains showed increases (compared to Hup^-) in weights of shoots, nodules and total plant material. This data was in agreement with earlier results obtained by Albrecht *et al.* (1979) which also showed that soybean plants inoculated with Hup^+ *R, japonicum* strains had more dry weight (16%) and N per total dry weight (10%) compared to plants inoculated with Hup^- strains. The results of both investigations indicate that, as suggested earlier by Dixon (1972), the capacity for recycling H_2 produced *via* nitrogenase could have some beneficial consequences in the plant-micoorganisms symbiosis. Dixon (1972) postulated three main advantages of an efficient H_2 recycling system to nitrogen fixing bacteria: (1) protecting nitrogenase from inhibition by O_2 by maintaining a low O_2 tension in the nodule through consumption of O_2 in the H_2 oxidation reaction, (2) oxidation of H_2 and thus preventing a build up of this gas in the nodule might also prevent inhibition of nitrogenase by H_2 and (3) recycling H_2 produced *via* nitrogenase to provide

reducing power for use in N_2-fixation and other processes. In the last 15 years, data supporting these proposed advantages have been obtained from several experiments carried out with different N_2-fixing organisms (e.g. Walker & Yates, 1978; Emerich *et al.*, 1979; Dixon *et al.*, 1981; Nelson & Salminen, 1982; Murry & Lopez, 1989).

In vitro state:

One of the processes in which this regained reducing power (proposal 3) might be used could be reduction of CO_2 under carbon-limiting conditions. For instance, Eisbrenner & Evans (1983) had speculated that since some species of *Xanthobacter* fix N_2 when grown chemolithotrophically with mineral salts, H_2, O_2 and CO_2, the possibility exists that electrons from H_2 are utilized first to reduce CO_2 to carbohydrates, the metabolism of which provides the reductant for N_2-fixation.

As already mentioned in the general introduction, CO_2 requirement for growth of five species (*B. japonicum*, *R. leguminosarum*, *R. meliloti*, *R. phaseoli* and *R. trifoli*) of rhizobia has been demonstrated by Lowe & Evans (1962). These authors showed not only a requirement of CO_2 for growth but also that none of various organic compounds suspected to be direct or indirect products of carboxylation reactions would replace or eliminate this CO_2 requirement. This information together with the data of Maier *et al.* (1978) formed the background for some work reported a year later by Hanus *et al.* (1979). These authors showed conclusively that in addition to the ability of all rhizobia to grow heterotrophically, some Bradyrhizobia species can grow autotrophically in culture. Autotrophic growth occurred only in those strains possessing hydrogen-uptake-positive capacity (Hup+) such as *B. japonicum* SR and wild type *B. japonicum* strains 136, 6 and 143, grown under an atmosphere of low-oxygen partial pressure, but of enriched H_2 and CO_2 content. Subsequently, Simpson *et al.*, (1979) reported that addition of H_2 to Hup+ *B. japonicum* SR increased the rate of CO_2 fixation by about 6-fold and that hydrogenase and ribulosebisphosphate carboxylase (RuBPc) are coordinately

induced by H_2 and are both repressed by succinate; similar responses under these conditions have been reported in the hydrogen bacterium *Aquaspirillum autotrophicum* (Aragno & Schlegel, 1978). Malik & Schlegel (1981) also reported autotrophic growth in *B. japonicum* strains DES 122, SR, and TAL 379 but failed to detect any activity of RuBPc in *R. leguminosarum* DSM 30143 and *R. trifoli* DSM 30141 strains.

The findings of Simpson *et al.*, (1979) were interpreted as suggesting that: (a) like certain hydrogen bacteria, the H_2-uptake positive strains of *B. japonicum* may have the ability to grow autotrophically - this conclusion was supported by the coupled expression of two enzymes required for the utilization of an inorganic energy source and for the fixation of CO_2 which suggested a conversion from heterotrophic growth to a mixotrophic or autotrophic type of metabolism and (b) the RuBP-dependent fixation of CO_2 in these strains under free-living conditions indicated a capability for fixing CO_2 through RuBPc, which presumably would participate in the ribulose-bis-phosphate pathway (the Calvin cycle). Confirmation of the function of this pathway in autotrophic cells of Hup+ strains of *B. japonicum* came from the enzymological studies of Lepo *et al.* (1980) which showed that 3-phosphoglyceric acid (PGA) was the product of RuBP-dependent fixation of CO_2, with a molar ratio of about 2 mol of PGA to 1 mol of CO_2 fixed. This is the first step in the Calvin cycle in the biosynthesis of cell biomass (see chapter 4).

3.1.2.2. *Frankia.*

Studies on the occurrence of uptake hydrogenase in actinorhizal symbioses are few compared with legume symbioses. Data available to date show that in contrast with most legume symbioses, almost all actinorhizal associations exhibit little or no H_2 evolution indicating the presence of an uptake hydrogenase (Moore, 1964; Schubert & Evans, 1976; Benson *et at.*, 1980; Winship & Tjepkema, 1983; Sellstedt &

Winship, 1984). Only two exceptions have so far been reported; one for *Ceanothus velutinus* (Schubert & Evans, 1976) and the other in a symbiosis between *A. incana* and a crushed nodule inoculum of *Frankia* from Sweden (Sellstedt & Huss-Danell, 1984),

Schubert & Evans (1976) surveyed H_2 evolution from excised nodules of legumes and actinorhizal plants. They found that H_2 from actinorhizal nodules was lost at a mean rate of only 4% of the nitrogenase electron flow, compared to a mean rate of 44% for legume nodules. Uptake hydrogenase activity has now been recorded in excised nodules (intact or homogenised) from several actinorhizal plants (Roelofson & Akkermans, 1979; Benson *et al.*, 1979,1980; Sellstedt & Winship, 1987; Winship *et al.*, 1987; Murry & Lopez, 1989; Sellstedt, 1989). Furthermore, in a more recent work Murry & lopez (1989) have shown that pure cultures of *Frankia* strain ArI3 also possess an uptake hydrogenase, under N_2-fixing conditions, and that H_2 supported about 30% of the respiratory activity observed with propionate in cells completely depleted of endogenous carbon reserves.

As already stated a requirement of CO_2 for growth of *Frankia* has been reported by Akkermans *et al.* (1983). This researcher showed that growth of *Frankia* AvcI1 on propionate as C-source was dependent on the presence of CO_2 since non-shaken cultures incubated in the absence of CO_2 gave significantly lower yields on propionate. This suggested that dark fixation of CO_2 is important in this organism, and may be for other strains too. Subsequently, Shipton & Burggraaf (1983) showed that when some *Frankia* strains were grown under near-chemoautotrophic conditions, *i.e*, the growth medium contained no C-sources apart from small amounts in the form of vitamins but the gas mixture over the cultures contained 10% each of H_2 and CO_2, the protein yield of these cultures was higher compared to cultures grown under atmospheric conditions. These findings together raised the possibility that, as in rhizobia, some *Frankia* species might be able to grow autotrophically *in vitro*. This possibility has been examined here.

3.2. MATERIALS AND METHODS.

3.2.1. Aseptic techniques.

One of the main reasons why the isolation and cultivation of *Frankia* can be difficult is that *Frankia* is a very slow-growing organism and can easily be out-grown by any contaminants present in the growth medium. Thus, in order to limit the chances of introduction of contaminants from the surrounding environment in the laboratory, the following aseptic techniques were employed at all times prior and during inoculation.

1- All growth media, flasks, culture-tubes, homogenisers, syringes and needles were heat-sterilized at 120 °C for 25 minutes.

2- When transferring sterile contents (media or mycelium in culture) from one container to an other, the mouths of both containers were flamed before and after transferring the contents.

3- To reduce air-borne contamination, the inoculation operations (step No 2) were performed within a laminar flow-hood.

3.2.2. Organisms.

The strains used in this study had been previously isolated from nodules of *Alnus* or *Myrica* and will be referred to hereafter according to a designation system used in this laboratory. The acronym used to designate each strain, identifies the host plant species and the individual isolation and is made of three numbers each separated by a decimal (Hooker, 1987). The first number indicates the genus: 1, *Alnus*; 3, *Myrica*. The second number indicates the species: 1.1, *Alnus glutinosa*; 1.2, *Alnus rubra* ..... and 3.1, *Myrica gale*. The third number indicates the isolation attempt followed by the isolation medium upon which the strain was first isolated. For example, 1.2.5Q is a strain isolated from *Alnus rubra* on Qmod medium during isolation attempt number (5). A list of all strains used here along with their more

common designation numbers according to the classification scheme of Lechevalier (1983) is given in the results section (Table 9).

3.2.3. Maintenance of cultures.

All stock cultures of *Frankia* were maintained in a defined medium referred to hereafter as BuCT medium and were routinely sub-cultured at two month intervals. After 7-8 weeks of standing culture, the cultures were aseptically harvested *in vacuo* on pre-sterilised Nalgene Analytical Filter Funnels (Nalgene Company, USA.. Type AF) and rinsed thoroughly with fresh medium. The mycelium was resuspended in BuCT medium and homogenised in a sterile Potter-Elvehjem homogeniser, and aliquots of the final homogenate were transferred to sterile culture-tubes (2 cm x 15 cm) containing about 15 ml. of medium. After inoculation, the tubes were plugged with cotton wool, covered with aluminium caps which were sealed to the tubes with "Nescofilm" and incubated in the dark at 27 °C, under non-shaking conditions.

The maintenance BuCT medium was a modification of the casamino Tween medium first used by Burggraaf and Shipton (1982). The basic composition was (grams / litre): $CaCl_2.2H_2O$, 0.1; $MgSO_4.7H_2O$, 0.2; casamino acids, 1.0; Fe Na EDTA, 0.01; biotin, 0.002; Na-propionate, 0.5 and Tween-80, 0.5. The pH was adjusted to 6.8. After this solution had been autoclaved and cooled it was aseptically mixed with a separately autoclaved phosphate buffer (pH 6.8) which contained K_2HPO_4, 1.0 $g.l^{-1}$; $NaH_2PO_4.2H_2O$, 0.67 $g.l^{-1}$ and trace elements as those of Baker and Torrey (1979), 1 $ml.l^{-1}$.

3.2.4. Preparation of inocula and quantification of *Frankia* cell biomass.

Prior to inoculation, experimental *Frankia* cultures - all of which were routinely maintained on BuCT medium as described in 3.2.3. - were aseptically harvested *in vacuo* on pre-sterilised Nalgene Analytical Filter Funnels (Nalgene Company, USA.. Type AF) and rinsed thoroughly with fresh carbon-deficient medium,

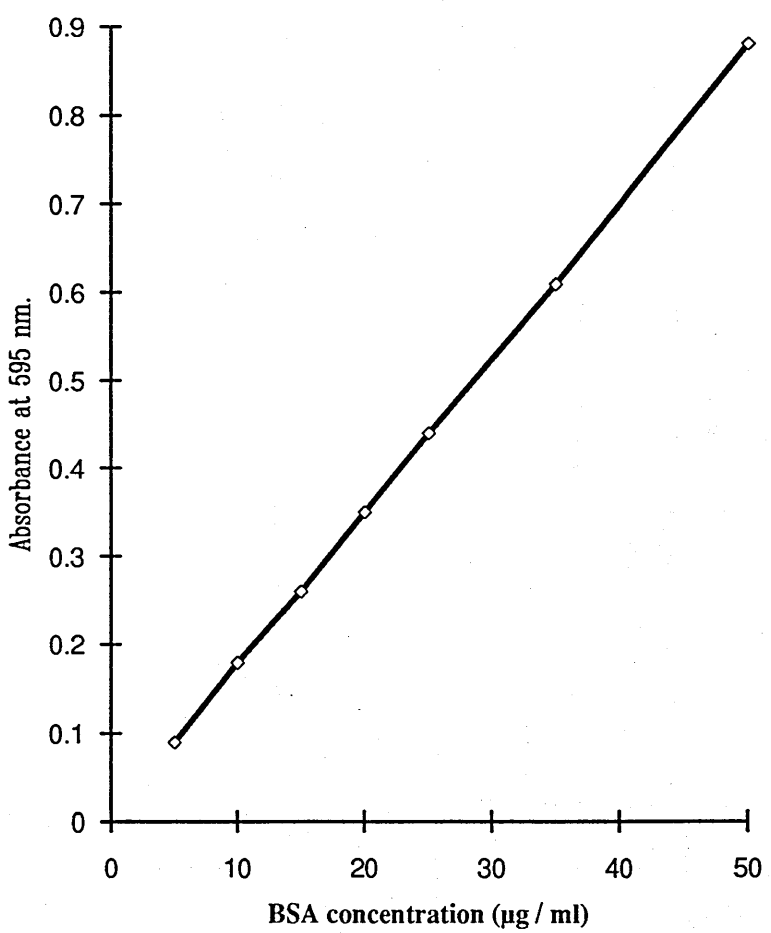
referred to hereafter as Bu-C, to remove all traces of carbon. The mycelium was resuspended in the desired fresh medium and homogenised by sucking it up into and then expelling it from a disposable plastic syringe fitted with a needle (gauge, 21). This operation was repeated until disruption was satisfactorily accomplished. The final homogenate was used to inoculate either glass-tubes (aliquots of 0.5 ml./10 ml growth medium) or flasks (aliquots of 1.5 ml./50 ml growth medium).

The experimental growth media used in the present study are referred to hereafter as (1) Bu medium, which had the same composition as BuCT except for the omission of Tween-80 and substitution of NH_4NO_3 (0.5g / l) for casamino acids and (2) Bu-C medium, which was as Bu except for the omission of the carbon source (i.e., Na-propionate). All cultures were incubated in the dark at 27 °C, under non-shaking conditions.

At harvest, growth of cultures was measured by assessing total protein using an assay based on the absorbance shift from 465 to 595 nm which occurs when Coomassie Blue G250 binds to proteins in an acidic solution (Bradford, 1976). Cultures were harvested by centrifugation in graduated tubes at 13,000 x g for 10 min at 4 °C, using an M.S.E, Mistral 2 L centrifuge, then decanting the medium. The resulting pellet was resuspended in distilled water, centrifuged as before and the supernatant decanted again. This operation was repeated three times to insure that no traces of the medium remained. The final pellet was resuspended in 3 ml. of distilled water and then disrupted by sucking it up and expelling it several times from a disposable plastic syringe fitted with a needle (gauge, 21). The resulting homogenate was sonicated for 1 min in an ice cooled stainless steel sonication chamber using an Ultrasonic, U.K Ltd. 180 G sonicator operated at full power (165 W). Hooker (1987) showed that no further protein release occurred after sonication of samples for 50 - 60 seconds under these conditions and this result was confirmed here prior to use.

Protein determinations were made on duplicate 1 ml homogenised and sonicated samples after mixing with 2 ml. of Coomassie blue G-250 Pierce Protein Assay Reagent (PPAR, product No. 23200) purchased from Pierce Chemical Company. Sample absorbance was measured at 595 nm using a spectrophotometer (Pye Unicam SP 8000) after standing for 10 min. at room temperature for full colour development, and protein content was determined by comparison with bovine serum albumin (BSA) standard curve (Sigma Chemical, Company Ltd), shown in Figure 6.

Figure 6: Calibration curve for reaction of bovine serum albumin (BSA) with Pierce protein assay reagent (PPAR).



A known protein concentration series within the range of 5 to 50 µg/ml was prepared by diluting a stock BSA standard in distiled water (the diluent used when sonicating *Frankia* mycelia for total protein determination). Duplicate 1.0 ml samples of the BSA standard diluents were pipetted into 16 x 100 ml glass- tubes. -The sample diluent (i. e., distiled water) was used as a blank. 2 ml of PPAR were then added per tube and mixed well. Absorbance values were measured at 595 nm, using a Pye Unicam SP 800 Spectrophotometer, corrected for blank and then plotted against BSA concentrations as shown on the graph.

3.2.5. Characterization of *Frankia* growth.

3.2.5.1. Growth rate.

A preliminary experiment was carried out to determine the growth rates of three *Frankia* strains, growing in the presence or absence of carbon sources. Stock cultures of the strains, all of which were routinely maintained previously on BuCT, were harvested as described in 3.2.4. The resulting mycelia were resuspended in Bu-C medium, homogenised as before and equal aliquots of inoculum homogenates were then used for inoculating glass-tubes (2 cm x 15 cm) containing 10 ml of either Bu or Bu-C medium. After inoculation, the tubes were covered with aluminium caps, sealed to the tubes with "Nescofilm", and incubated in the dark under non-shaking conditions at 27 °C.

Growth was assessed weekly over a period of 7 weeks by harvesting the contents of 5 tubes of each set. Harvest, sonication and determination of protein content procedures were as described in 3.2.4.

3.2.5.2. Carbon and vitamin requirements for growth.

The same strains used in the previous experiment were again used here to (a) test their ability to utilize a number of carbon substrates for growth, (b) determine if biotin is necessary for their growth and (c) assess the influence of ambient CO_2 over the cultures on their growth in the presence of propionate.

The strains tested, all of which were routinely maintained previously on BuCT, were harvested and homogenised as before and then dispensed into 250-ml conical flasks containing 100 ml of Bu-C medium. The flasks, plugged with cotton wool and closed with aluminium foil, were incubated in the dark at 27 °C, under non-shaking conditions.

Two weeks later, starved cultures were filtered and the resulting mycelia resuspended in Bu-C medium deficient in biotin, homogenised as before and equal aliquots of inoculum homogenates were used for inoculating glass-tubes (2 cm x 15

cm) containing 10 ml of the desired growth medium. In the carbon substrate utilization experiment, propionate was omitted from Bu medium and various carbon substrates (glucose, succinate or pyruvic acid) were substituted. In the experiment designed to determine if biotin was necessary for *Frankia* growth, the growth medium was either Bu-C or Bu, containing different concentrations of biotin which were added to the media by filter sterilisation. In these two experiments, inoculated tubes were covered with aluminium caps which were sealed to the tubes with "Nescofilm" and incubated in the dark under non-shaking conditions at 27 °C.

In the experiment designed to assess the influence of ambient CO_2 over the cultures on *Frankia* growth, glass-tubes (2 cm x 15 cm) containing 10 ml. of Bu medium were inoculated, plugged with cotton wool, and then randomised within 1.4 l-capacity glass jars with lids fitted with rubber stoppers to facilitate adjustment of the gas mixtures within the jars after closure. The latter step was done by withdrawing, using a syringe, calculated volumes of air present in the incubation jars and replacing them with equivalent volumes of CO_2 to obtain the desired gas mixture within each jar (1, 3, 5, or 10% CO_2, with air making up the balance). The gas mixtures over the cultures were changed and adjusted in the same manner twice a week. The cultures were incubated in the dark at 27 °C, under non-shaking conditions.

3.2.6. Autotrophic growth of *Frankia*.

A number of strains were examined for their ability to utilize (a) microbial reserves, or (b) $CO_2 + H_2$ as sole carbon and energy sources.

Frankia strains tested, all of which were routinely maintained previously on BuCT, were harvested and homogenised as described in 3.2.4. then dispensed into 250-ml conical flasks containing 100 ml of Bu-C medium. The flasks, plugged with cotton wool and closed with aluminium foil, were incubated in the dark at 27 °C,

under non-shaking conditions.

Two weeks later, starved cultures were filtered, homogenised and aliquots of 0.5 ml were transferred to culture-tubes (2 cm x 15 cm) containing 10 ml of Bu-C medium.

In the experiment designed to test the ability of *Frankia* to grow on microbial reserves, inoculated culture-tubes were covered with aluminium caps which were sealed to the tubes with "Nescofilm". In the experiment designed to test the ability of *Frankia* strains to grow on CO_2 and H_2, inoculated culture-tubes were plugged with cotton wool, and then randomised within glass containers fitted with rubber stoppers to facilitate adjustment, twice weekly, of the gas mixtures over the cultures. The containers were flushed with N_2 and allowed to equilibrate in air for 20 minutes before adjusting the gas mixtures within the closed containers to the desired compositions (5% H_2 + 5% CO_2 + different O_2 concentrations with N_2 making up the balance) using a Pye Unicam Series 104 Gas Chromatograph operated isothermally (60 °C) with a 1 metre x ¼ inch steel column of Poropack N (100-120 mesh), a carrier gas of oxygen-free nitrogen (flow rate, 35 $ml.min^{-1}$) and flame ionisation detector (hydrogen/air flame). This was done by first determining the [O_2] within each container by comparing the peak given by a 0.5 ml sample withdrawn from air within the container and injected into the GC, with the peak given by a 0.5 ml air sample from the laboratory. The calculated required amounts of O_2, H_2 and CO_2 to achieve the desired gas mixtures were added, using syringes. All gases used in this experiment were obtained in cylinders from the British Oxygen Company, Special Gases, Ltd.

All cultures were incubated in the dark at 27 °C, under non-shaking conditions.

3.2.7. Measurement of activities of carboxylase enzymes in cell-free extracts of propionate-grown *Frankia.*

3.2.7.1. Growth of organisms:

Frankia strains, all of which were maintained previously on BuCT medium, were harvested and homogenised as described in 3.2.4. Homogenate aliquots of 1 ml were added to 100-ml conical flasks containing 50 ml of Bu medium. The flasks, plugged with cotton wool, were randomised within glass containers and CO_2 added, after closing the containers, to a final concentration of 5 % of the atmosphere within each container. The atmospheres (95% air + 5 % CO_2) over the cultures were changed twice weekly.

3.2.7.2. Preparation of cell-free extract:

After 7 weeks growth, cultures were filtered on 0.2 µm Millipore membrane filters (47 mm diameter) and the mycelium washed with 0.1 M tricine buffer, pH 7.5, containing 2 mM reduced glutathione. The cells were resuspended in buffer, sonicated for 3 minutes at full power and then centrifuged at 13,000 x g for 20 min. at 0 °C. The supernatant liquid was removed and stored in ice until used for carboxylase assays.

3.2.7.3. Carboxylase assays:

Assays for activities of five different carboxylase enzymes were performed by published methods: RuBP carboxylase, Propionyl CoA carboxylase, PEP carboxylase and PEP carboxykinase (Simpson et al., 1979), and Pyruvate carboxylase (Utter & Keech, 1963).

Each reaction mixture (1 ml volume) contained the following additions in µmol unless otherwise indicated. Tricine buffer, pH 8.0, 100; $MgCl_2.6H_2O$, 5; Na_2 EDTA, 0.03; reduced gluthatione, 3; *Frankia* extract, 0.4 ml containing about 0.15

µg protein; $NaH^{14}CO_3$, 35.7 containing 0.07 µCi µmol^{-1}. The following substrates in µmol were added to the assays for specific enzymes: **RuBP carboxylase**, RuBP, 0.25; **Propionyl CoA**, Prop CoA, 0.25 and ATP, 5; **PEP carboxylase**, PEP, 0.25; **PEP carboxykinase**, PEP, 0.25 and Na_2 ADP, 5; **Pyruvate carboxylase**, Na-pyruvate, 10, ATP, 25 and acetyl CoA, 0.7. Either enzyme substrates or *Frankia* cell-free extracts were omitted from control reactions. Before adding substrates, reaction mixtures were preincubated in a water bath at 30 °C for 10 min. Substrates were then added and the incubation continued for 30 min. Reactions were terminated by the addition of 2 % 2,4-dinitrophenyl in 2 N HCl then incubated for 3 hours at room temperature to remove excess $^{14}CO_2$. Aliquots (200 µl) of terminated reaction mixtures were transferred to scintillation vials, Ecoscint (National Diagnostics, UK) was added and the samples were assayed for radioactivity using an LKB 1211 Minibeta Liquid Scintillation Counter. Net counts per minute (cpm) were converted to disintegrations per minute (dpm) by the channels ratio method.

3.3. RESULTS.

3.3.1. Characterization of *Frankia* growth.

3.3.1.1. Growth rate.

From the growth rates presented in Figure 7 it can be concluded that all three strains tested continued to grow slowly, under the conditions used for 5 to 6 weeks when a stationary phase was reached followed by a decline (autolysis) phase.

3.3.1.2. Carbon and vitamin requirements for growth.

The influence of a number of carbon substrates and biotin (the only added vitamin in the Bu growth medium) on growth of three *Frankia* strains are summarised in Tables 6 and 7, respectively.

Total protein yields shown in Table 6 demonstrate that none of the three carbon substrates (glucose, succinate or pyruvic acid) substituted for propionate in the growth medium supported a level of growth statistically significant even at $P \leq 0.15$. All strains grew well on propionate medium.

Table 6. Growth yield (µg protein / 10-ml cultures)[1] of three *Frankia* strains grown on various carbon sources for 40 days.

strain	Carbon source (0.5g / l)				
	Control[2]	Propionate	Glucose	succinate	Pyruvic acid
ArI4	11.0±4.37	107.2±3.53	12.0±1.74	21.3±4.76	12.3±1.98
1.2.5Q	10.8±0.58	90.9±4.74	11.42±1.17	14.5±1.94	12.5±2.65
1.1.14Q	13.8±0.93	112.6±6.55	15.52±2.31	13.4±1.31	16.2±1.86

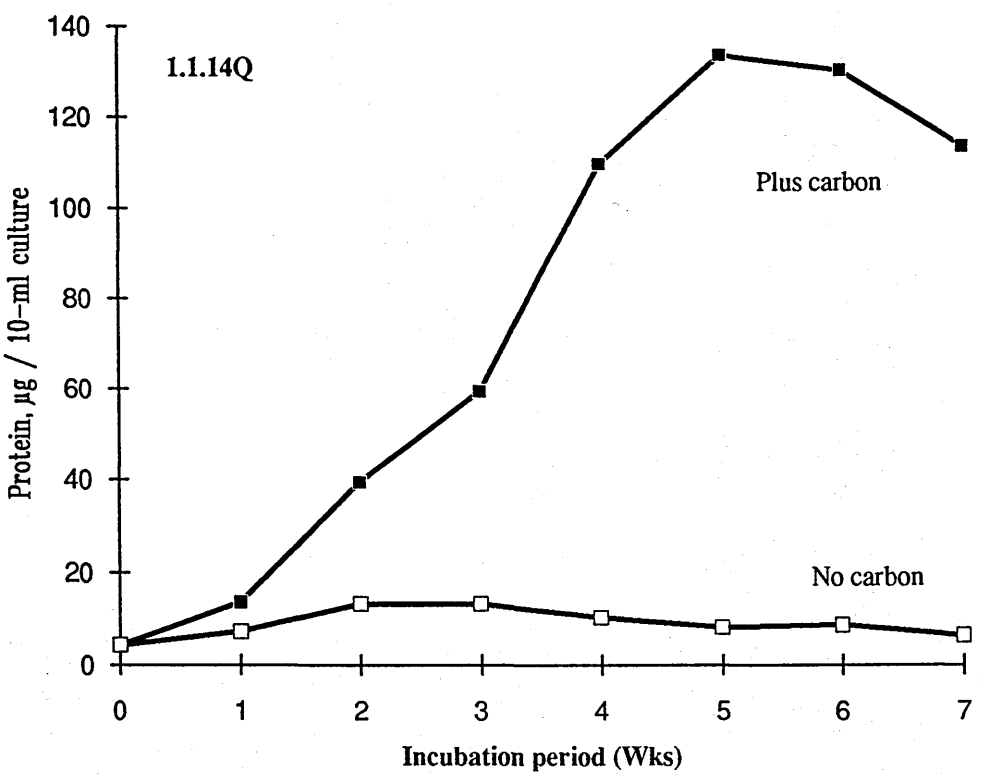
[1] Mean ± SE (n = 5).

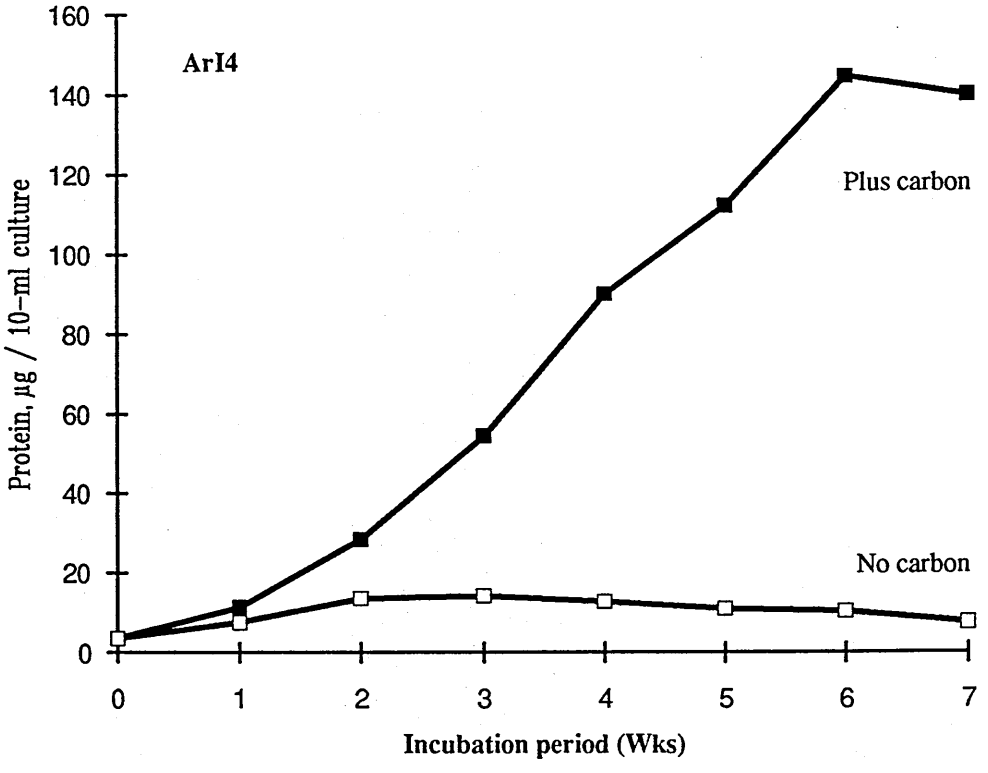
[2] Carbon-deficent medium (Bu-C).

Figure 7. Growth of three *Frankia* isolates (1.1.14Q, ArI4 and 1.2.5Q) in the presence and absence of Na-propionate (0.5g / l) as carbon source.

Frankia mycelium homogenates were prepared as described in 3.2.4 and used to inoculate glass-tubes (2 cm x 15 cm) each containing 10 ml of the desired medium. After inoculation, tubes were covered with aluminum caps, which were sealed to the tubes with "Nescofilm", and incubated in the dark under non-shaking conditions at 27 °C.

Growth was assessed weekly over a period of 7 weeks by harvesting the contents of 5 tubes of each set and determining total protein content as described in 3.2.4.




ArI4
Plus carbon
No carbon
Protein, µg / 10-ml culture
160
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Incubation period (Wks)

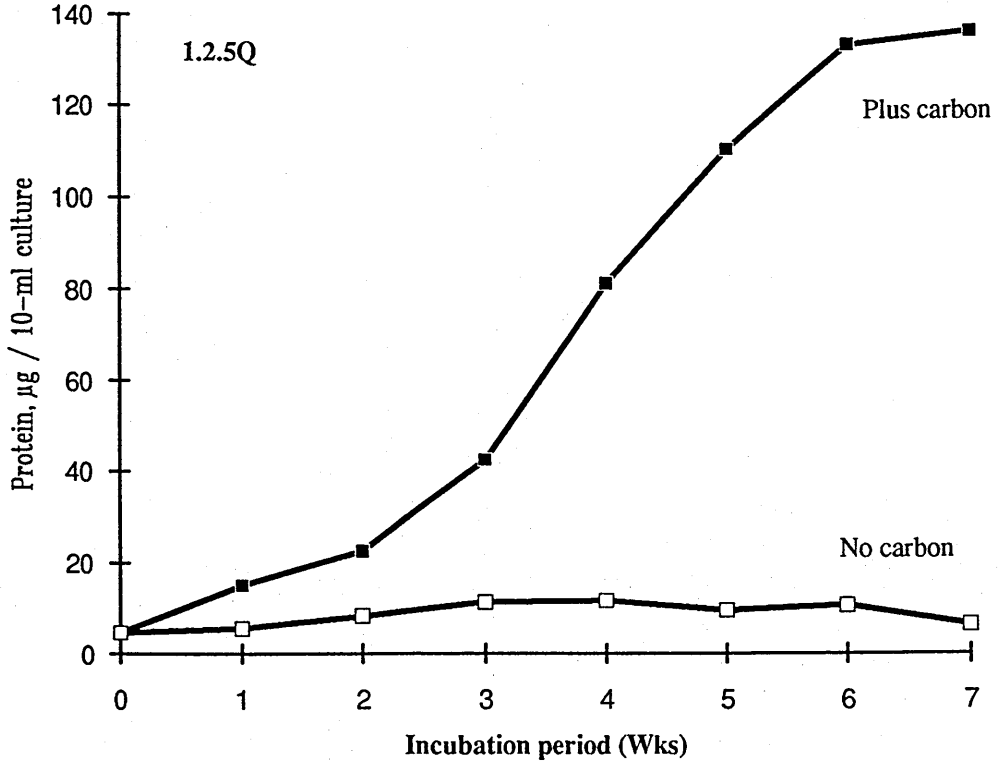

1.2.5Q
Plus carbon
No carbon
Protein, µg / 10-ml culture
140
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Incubation period (Wks)

Table 7 shows that addition of biotin to the growth medium had no statisticall significant effect on growth (protein yield) of all *Frankia* strains tested.

Table 7. Growth yield of *Frankia* strains after cultivation for 42 days in Bu media containing various concentrations of biotin.

Growth medium	Biotin (mg / l)	Protein yield (µg / 10-ml culture)[1]		
		ArI4	1.2.5Q	1.1.14Q
Bu - C	0	8.9 ± 0.59	7.7 ± 0.57	13.4 ± 0.48
"	2	10.8 ± 1.69	9.6 ± 0.92	15.9 ± 1.48
Bu	0	128.3 ± 7.17	93.0 ± 8.85	98.8 ± 8.18
"	2	137.9 ± 6.42	98.5 ± 5.13	109.2 ± 7.59
"	10	139.5 ± 6.02	99.8 ± 4.17	115.7 ± 5.66
"	50	141.4 ± 6.04	101.3 ± 5.32	118.4 ± 8.76
"	100	137.7 ± 6.44	105.2 ± 7.93	108.5 ± 7.31

[1], Mean ± SE (n = 5).

The effect of ambient CO_2 over the cultures on *Frankia* growth also has been examined here. The data obtained from this experiment is summarised in Table 8 which shows that the protein yield of ArI4 and 1.2.5Q was increased by 18 and 22%, respectively, as the ambient CO_2 was increased from air levels (0.03%) to 3%. Further increase of the ambient CO_2 to 5 and 10% did not result in any additional significant increase in protein yield of either strain. The 5% CO_2 level was selected for future work in experiments concerned with autotrophic growth and enzymic assays.

Table 8. Growth yield of two *Frankia* strains cultivated in Bu medium under an atmosphere enriched with different CO_2 concentrations for 40 days.

strain	Growth yield (µg protein / 10-ml culture)[1]				
	Air control	1% CO_2	3 % CO_2	5% CO_2	10 % CO_2
ArI4	106.8±2.09	116.6±1.95	126.1±1.97	129.5±173	127.9±4.68
1.2.5Q	92.0±3.46	100.9±2.86	111.6±2.61	117.0±2.42	120.0±5.44

The strains were cultivated in glass tubes each containing 10 ml of Bu medium. The culture-tubes, plugged with cotton wool, were placed inside1.4-litre screw-cap jars with lids fitted with rubber stoppers to facilitate adjustment, twice weekly, of CO_2 concentrations in the jars as described in Materials and Methods.

[1]Data are means ± SE, (n = 5).

3.3.2. Autotrophic growth of *Frankia*.

Data obtained from studies concerned with the ability of a number of *Frankia* strains to utilize (a) microbial reserves or (b) CO_2 + H_2 as carbon and energy sources for growth are shown in tables 9 and 10, respectively.

Both sets of data suggest that none of the strains tested can utilize microbial endogenous reserves or inorganic compounds as carbon and energy sources.

Table 9. Protein yield (µg/10-ml culture)[1] of some *Frankia* strains grown in the presence and absence of carbon source (Na-propionate, 0.5 $g.l^{-1}$) for 6 weeks.

strain		Growth medium[2]	
Acronym	Catalogue number	Bu	Bu-C
Alnus rubra strains:			
ArI3	HFP 01 31 01 10	120.6 ± 1.72	12.3 ± 3.66
ArI4	DDB 01 31 02 10	165.4 ± 2.97	11.7 ± 1.18
ArI5	DDB 01 31 03 10	86.2 ± 4.24	9.9 ± 1.60
1.2.5Q	UGL 01 31 03	144.2 ± 3.99	11.9 ± 2.29
1.2.13Q	UGL 01 31 06	99.9 ± 4.04	9.82 ± 1.00
1.2.19Q	UGL 01 31 10	134.6 ± 2.76	10.27 ± 0.9
1.2.23Q	UGL 01 31 13	117.9 ± 2.61	6.78 ± 0.55
ALnus glutinosa strains:			
1.1.1Bu	UGL 01 07 01	79.6 ± 4.17	7.58 ± 0.90
1.1.5F	UGL 01 07 04	33.2 ± 2.25	9.77 ± 0.59
1.1.7F	UGL 01 07 06	31.8 ± 3.61	5.8 ± 0.93
1.1.8Bu	UGL 01 07 08	116.6 ± 6.93	8.98 ± 1.095
1.1.14Q	UGL 01 07 10	95.9 ± 1.04	10.58 ± 0.472
Myrica gale strains:			
3.1.5P	UGL 16 11 01	25.8 ± 3.02	6.64 ± 0.622
3.1.10P	UGL 16 11 02	46.4 ± 3.55	7.36 ± 0.460

1, Mean ± SE (n = 5).

2, The cultures were grown in glass-tubes each containing 10 ml medium inoculated with 6 to 12 µg protein inoculum.

Table 10. Growth yield of *Frankia* strains after cultivation for 6 weeks under autotrophic conditions.[1]

strains	Growth yield (µg protein / 10-ml culture)[2]				
	control[3]	1.5% O_2	5% O_2	10% O_2	20% O_2
ArI3	17.3 ± 0.90	16.0 ± 0.67	15.7 ± 1.52	18.4 ± 2.31	18.3 ± 1.23
ArI4	13.5 ± 2.14	14.2 ± 0.95	16.8 ± 2.10	13.4 ± 1.65	12.3 ± 1.45
ArI5	16.7 ± 2.81	15.7 ± 0.24	11.8 ± 3.20	15.3 ± 2.30	13.3 ± 3.21
1.2.5Q	7.3 ± 2.25	8.7 ± 1.35	6.0 ± 1.45	7.4 ± 3.20	11.0 ± 3.28
1.1.5F	9.6 ± 3.90	11.6 ± 0.85	8.4 ± 2.35	13.2 ± 1.76	11.2 ± 1.50
1.1.7F	6.4 ± 0.50	5.5 ± 2.40	8.6 ± 1.12	7.4 ± 0.65	9.4 ± 2.45
1.1.14Q	12.5 ± 1.65	13.0 ± 1.14	9.6 ± 2.65	11.0 ± 0.87	14.2 ± 1.60
3.1.5P	11.6 ± 1.24	10.6 ± 1.87	15.0 ± 2.06	11.4 ± 0.85	13.2 ± 1.75
3.1.10P	7.3 ± 2.06	9.2 ± 0.67	6.3 ± 1.62	10.1± 0.85	8.4 ± 2.24

[1] The strains were grown in glass-tubes each containing 10-ml of Bu-C medium. After inoculation, the tubes were plugged with cotton wool and placed inside glass containers fitted with rubber stoppers to facilitate adjustment of the gas mixture over the cultures (5% H_2 + 5% CO_2 + different O_2 concentrations with N_2 making up the balance), twice weekly, as described in 3.2.6.

[2] Data are mean ± SE, (n = 5).

[3] The control cultures were grown in Bu-C but under normal atmospheric conditions. The average growth yield of cultures (one 10-ml tube per strain) also grown under normal atmospheric conditions, but with propionate added to the medium was 119.17 ± 12.50 µg protein .

3.3.3. Carboxylase assays:

The enhancement of growth of *Frankia* strains ArI4 and 1.2.5Q by up to 22% following enrichment of the air in which the strains were maintained with 3% CO_2 (Table 8), indicates the presence of substantial carboxylase activity in these strains. Data obtained from assays for activities of 5 different carboxylase enzymes in cell-free extracts of these strains are shown in Table 11. It can be seen that the main carboxylase activities detected were PEP carboxylase and PEP carboxykinase, with lesser activity of pyruvate carboxylase and propionyl CoA carboxylase activity. Ribulose-bis-phosphate carboxylase (RuBPc) was not detected.

Table 11. Activity of carboxylase enzymes of *Frankia* cells grown on Bu medium in air supplemented with 5 % CO_2.

Enzymes	Fixation of $^{14}CO_2$ (dpm . min^{-1}. mg^{-1}protein)			
	ArI4		1.2.5Q	
	A	B	A	B
RuBP carboxylase	ND	ND	ND	ND
Propionyl CoA carboxylase	ND	24.75	ND	104.2
PEP carboxylase	3458.2	3613.1	885.5	230.71
PEP carboxykinase	3402.4	3558.9	262.2	488.11
Pyruvate carboxylase	112.5	263.3	NA	NA

ND = not detected. NA = not assayed.

The rates of $^{14}CO_2$ fixation shown are means of duplicate assays of each enzyme. The data were corrected for: A, fixation obtained in controls from which the enzyme substrates were omitted and B, fixation obtained in the controls from which *Frankia* extracts were omitted.

3.4. DISCUSSION

Data obtained by other researchers working with *Frankia in vitro* prior to the commencement of this study demonstrated (a) the requirement of CO_2 for growth (Akkermans *et al.*, 1983) and (b) that in the absence of organic carbon compounds, H_2 and CO_2 can serve as sources of energy and carbon, respectively (Shipton & Burggraaf, 1983). It was the aim of this part of the present study to investigate these findings further, using a larger number of strains.

3.4.1. Characterization of *Frankia* growth.

The growth of *Frankia* under the culture conditions used in the present experiments was similar to that reported in the literature. Although the doubling time of protein was not determined, it can be calculated from the growth curves shown in Figure 7 that the three strains have a doubling time in the range of 3 to 4.5 days which falls within the range of 2 to 5 days reported by Diem & Dommergues (1983).

Most published work concerning carbon substrate utilization by *Frankia in vitro* indicates that propionate is almost a universal carbon source for growth. This conclusion is supported by the data presented in Table 6. The failure of all three strains to utilize glucose, succinate or pyruvic acid (Table 6) is also in agreement with previous studies (Blom *et al.*, 1980; Shipton & Burggraaf, 1982,1983; Akkermans *et al.*, 1983; Lopez *et al.*, 1986; Zhang *et al.*, 1986) which showed that not all *Frankia* strains utilize these three compounds, when provided singly, as carbon source. Nevertheless, many *Frankia* strains have been shown to grow satisfactorily on the organic acids succinate and pyruvic acid. The failure of our strains to utilize these compounds could be due to the pre-growth conditions of the inoculum (the stock cultures were maintained on BuCT which contained propionate and Tween-80 as C-sources) since the studies cited before showed that some strains can adapt to grow on C-substrates they do not usually use. The inability to utilize

glucose is less surprising since all 3 strains tested belong to group B (see page 75), which prefer short chain fatty acids or TCA intermediates as carbon source. It must be noted, however, that in contrast to the present data Hooker (1987) showed growth of 1.2.5Q on succinate and of ArI4 on glucose as sole C-source, despite maintaining the stock cultures on BuCT. This difference between the two sets of data could be due to (a) the extensive washing of mycelia, employed in the present study to remove all traces of propionate and Tween-80, and (b) starvation of *Frankia* cultures for two weeks on Bu-C before transfer to the experimental growth media. Nevertheless, the possibility remains that these strains may have lost the uptake system for these compounds or some enzymes needed for their degradation during their continuous culture since isolation in the early 80's. Furthermore, studies have also shown that some compounds can be utilized only when an additional carbon source (e.g., Tween-80) is present in the growth medium (Blom *et al.*, 1980; Blom, 1982; Lechevalier & Ruan, 1984; Hooker, 1987).

The data on the influence of biotin on *Frankia* growth (Table 7) is of particular interest and can be interpreted to suggest two possibilities. On the one hand, the failure of the 3 strains to respond significantly to biotin addition to the growth medium may indicate a synthetic ability for this vitamin. On the other hand, the apparent increase in protein yield of propionate-grown cells is an indication that propionate is catabolized *via* the methylmalonyl pathway, which involves the carboxylation of propionate by the action of the biotin-dependent propionyl-CoA carboxylase enzyme on propionyl-CoA to produce (s) methylmalonyl-CoA (Stowers *et al.*, 1986). The latter possibility is supported by CO_2-dependence of growth when propionate was the carbon source (Table 8) and by the presence of propionyl-CoA carboxylase in cell-free extracts (Table 11).

3.4.2. Autotrophic growth of *Frankia*.

The failure of carbon-starved strains to grow in air supplemented with 5% H_2 + 5% CO_2 and 1.5, 5, 10 or 20% O_2 (Table 10) indicate that these strains can not grow autotrophically. Simpson *et al.*, (1979) showed that in an experiment where 5% CO_2 was supplied in air over cultures, to make-up for the absence of organic carbon in the growth medium, addition of H_2 to autotrophic cells of Hup+ *B. japonicum* SR increased the rate of CO_2 fixation by about 6-fold. Murry & Lopez (1989) also demonstrated that H_2 supported about 30% of the respiratory activity observed with propionate in ArI3 *Frankia* cells completely depleted of endogenous carbon reserves. Therefore, it is possible that the autotrophic growth of some *Frankia* strains reported by Shipton and Burggraaf (1983) could be due to metabolism of traces of carbon from compounds, residual in the growth medium, which was enhanced by the presence of H_2 and CO_2 in air over the cultures. This was not possible in the present experiments because of, as already mentioned, the extensive washing of mycelia and starvation of cultures in Bu-C before transfer to the experimental growth medium.

The presence of substantial carboxylase activities in ArI4 and 1.2.5Q strains was indicated by enhancement of growth (protein yield) by 18 and 22%, respectively, following enrichment of the air in which the strains were maintained with 3% CO_2 (Table 8). It seemed essential, therefore, to examine these strains for enzymes considered most likely to participate in CO_2 fixation reactions. Simpson *et al.*, (1979) concluded from their studies that the RuBP-dependent fixation of CO_2 in autotrophic cells of Hup+ strains of *B. japonicum*, under free-living conditions, was indicative of CO_2 fixation by RuBPc, which presumably would participate in the ribulose-bis-phosphate pathway (the Calvin cycle). Their conclusion was confirmed by the enzymological studies of Lepo *et al.* (1980).

The failure to detect RuBPc in cell-free extracts of both *Frankia* strains tested here (Table 11) is conclusive evidence that these strains lack the ability to fix

CO_2 *via* the ribulose-bis-phosphate pathway. CO_2 is probably fixed *via* propionyl CoA carboxylase, which showed some activity in both cell-free extracts. The findings that both cell-free extracts of free-living strains contained activity of PEP carboxylase raise the question of whether free-living *Frankia* fixes carbon by this route. In the symbiotic state most studies showed that in both actinorhizal and legume nodules most if not all PEPc activity is associated with host plant cell cytoplasm (Christeller *et al*, 1977; Perrot-Rechenmann *et al.*, 1981; Vidal *et al.*, 1986; Miller *et al.*, 1987; Suganuma *et al.*, 1987). However, as already discussed in the Introduction to chapter 2, there has been controversy concerning the occurence and expression of activity of this enzyme in bacteroids.

CHAPTER FOUR:

C_1 COMPOUNDS UTILIZATION BY *FRANKIA*.

4.1. INTRODUCTION.

As defined in the general introduction, C_1-compounds are organic compounds that are more reduced than CO_2 and contain no carbon-carbon bonds, although some contain more than one carbon atom (Large, 1983). The ability of many micro-organisms to use these compounds, as sole carbon and energy sources for growth, has been the focus of much research carried out by both microbial physiologists and biochemists for several decades. Such micro-organisms are now termed methylotrophs (Large & Bamforth, 1988).

Methylotrophs include a wide variety of microorganisms- bacteria, yeasts and some filamentous fungi. Some of these organisms metabolize their growth substrates aerobically, others do so anaerobically. However, since *Frankia*, the organism studied here, is an aerobic bacterium; only aerobic bacterial methylotrophs will be considered.

4.1.1. Classification of aerobic methylotrophic bacteria.

Physiologically, aerobic bacterial methylotrophs can be divided into two main groups (Table 12). The first group consists of facultative methylotrophs which, in addition to their ability to grow methylotrophically on reduced C_1-compounds, also have the capacity to grow heterotrophically or autotrophically. Thus, this group itself can be divided into two sub-groups. (A) Autotrophic methylotrophs, which oxidize their reduced C_1 growth substrates to CO_2 then use the generated energy and reducing power to fix the CO_2 *via* the Calvin cycle (ribulose bisphosphate cycle). This subgroup consists mainly of chemoautotrophic organisms which, in addition to using C_1 compounds, also have the ability to obtain energy by the oxidation of inorganic compounds like dihydrogen (H_2) or reduced sulphur and use it to fix CO_2 *via* the Calvin cycle. Some photosynthetic bacteria are capable of

oxidizing C_1 compounds under aerobic conditions as well as growing heterotrophically and they, therefore, also fall into this sub-group. (B) Heterotrophic methylotrophs, which can grow heterotrophically on multicarbon compounds as well as using reduced C_1 compounds as sole carbon and energy sources. They are neither photosynthetic nor chemoautotrophic. They assimilate all or some of their carbon at the oxidation level of formaldehyde when growing methylotrophically.

The second group consists of obligate methylotrophs which can only grow on reduced C_1 compounds and on no other carbon source. The reason for obligate methylotrophy seems to be the lack of the enzyme 2-ketoglutarate dehydrogenase of the TCA cycle, preventing the functioning of the cycle and thereby preventing growth on substrates whose metabolism require operation of the cycle, i.e., multicarbon compounds (Large & Bamforth, 1988). This suggestion is supported by the data of Taylor & Anthony (1976) who found that mutants of the facultative heterotrophic methylotroph *Pseudomonas* AM1, lacking 2-ketoglutarate dehydrogenase, failed to grow on multicarbon compounds.

Biochemically, since autotrophic fixation of CO_2 by both plants and bacteria has been known for a long time, it is not surprising that early suggestions as to the pathways employed by bacteria when assimilating reduced C_1 compounds tended to focus on those involved in autotrophic growth, i.e., oxidation of the substrate to CO_2 followed by CO_2 assimilation *via* the Calvin cycle (Van Niel, 1954). Detailed studies in the last three decades, however, have shown that while this pathway is employed by some bacteria (autotrophic methylotrophs), there are now two other well defined pathways for C_1 assimilation in which the carbon is assimilated either as formaldehyde (i.e., the hexulose mono-phosphate pathway; HMP) or as a mixture of formaldehyde (50% to 70%) and CO_2, but *via* reactions distinct from those of the Calvin cycle (i.e., the serine pathway) (see Table 12).

Table 12. The physiological and biochemical classification of aerobic methylotrophic bacteria.[1]

Physiological group.	Carbon assimilation pathway.	Examples.	C_1 compound(s) used.
I- Facultatives:			
A- Autotrophs.	Calvin cycle.	Chemoautotrophs:	
		Micrococcus denitrifcans.	Methanol, Formate.
		Microcyclus aquaticus.	Methanol, Formate.
		Paracoccus denitrificans.	Methanol, Formate, Methylamines.
		Pseudomonas carboxydovorans.	Carbon monoxide.
		Pseudomonas gazotropha.	Carbon moxide.
		Thiobacillus novells.	Formamide.
		Phototrophs:	
		Rhodopseudomonas acidophila.	Methanol, Formate.
		Rhodopseudomonas polustris.	Formate.
		Others:	
		Bacterium sp. 7d.	Methylamines.
		Pseudomonas oxalaticus.	Formate.
B-Heterotrophs.	1- HMP.	*Arthrobacter* P1.	Methylamines.
		Arthrobacter globiformis.	Methylamines.
		Bacillus spp.	Methylamines.
		Brevibacterium sp. 24.	Methylamines.
		Mycobacterium vaccae 10.	Methanol.
		Nocardia sp. 239 .	Methanol.

Continued on next page. .....

Table 12. Continued.

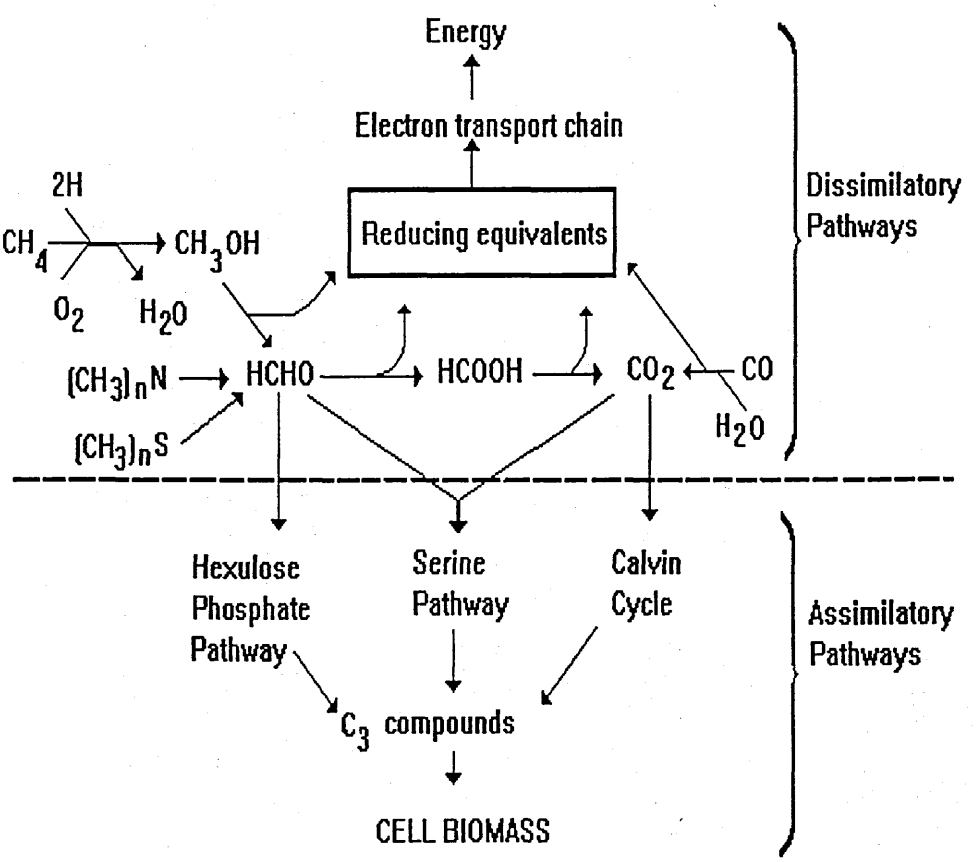
	2- Serine pathway.	*Bacterium* 5B1.	Methylamines.
		Methylobacterium organophilum.	Methane, Methanol.
		Pseudomonas AM1.	Methane, Methanol, Methylamines.
		Pseudomonas MA.	Methylamines.
		Pseudomonas aminovorans.	Methylamines.
II- Obligates:	1- HMP.	*Methylobacter capsulatus.*	Methane.
		Methylococcus capsulatus.	Methane, Methanol.
		Methylomonas methanica.	Methane, Methylamines.
		Methylophilus methylotrophus.	Methane, Methylamines.
		Pseudomonas C.	Methanol.
		Pseudomonas W1.	Methylamines.
		Pseudomonas W6.	Methanol, Formaldehyde.
	2- Serine pathway.	*Methylocystis.*	Methane.
		Methylomonas methanooxidans.	Methane, Methanol.
		Methylosinus trichosporium.	Methane, Methanol.

[1] Complied from Colby *et al.* (1979), Hanson (1980); Large & Bamforth (1988) and Dijkhizen & Arfman (1990).

4.1.2. Metabolism of C_1 compounds by aerobic methylotrophic bacteria.

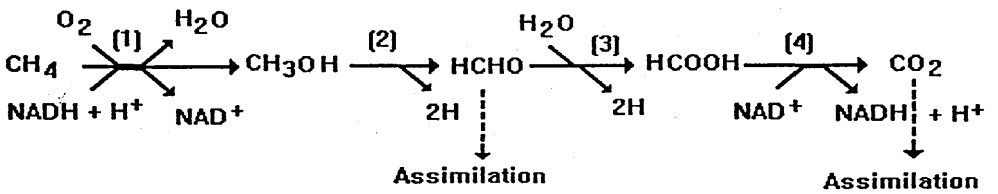
The assimilation of reduced C_1 compounds by the methylotrophic cell is energy dependent. The energy and reducing power for these assimilatory reactions are provided by the oxidation of the reduced C_1 growth substrates themselves. Thus, the metabolic transformations of C_1 compounds in methylotrophic cells can be divided into two stages (Fig. 8): (1) the dissimilatory pathways by which the various C_1 growth substrates are oxidized to CO_2, thereby making both energy and reducing power available to the cell and (2) the assimilatory pathways by which carbon enters cell material.

Figure 8. Growth of bacteria on reduced C_1 compounds (Large, 1983).



4.1.2.1. Dissimilatory pathways.

All bacteria oxidizing methane or methanol do so *via* the following route:



Key enzymes: (1), methane mono-oxygenase; (2), methanol dehydrogenase; (3), formaldehyde dehydrogenase and (4), formate dehydrogenase.

4.1.2.1.1. Oxidation of methane.

Söhngen (1906) was the first to isolate an organism able to grow on methane as the sole carbon and energy sources. He named the bacterium *Bacillus methanicus*. However, despite the widespread natural occurrence of methane, apart from this bacterium which was re-isolated and renamed on several occasions [e.g., as *Pseudomonas methanica* (Dworkin & Foster, 1956) and then as *Methylomonas methanica* (Foster & Davis, 1966)], only three other new species were isolated in pure culture in the period between 1906 and 1966 (Quayle, 1972). The situation has changed following the improvement of techniques for enrichment, isolation and culture of methane-oxidizers by Whittenbury *et al.* (1970) who were able to isolate over a 100 of new methane-utilizing strains.

It is well established that the first step in methane oxidation is catalysed by the methane mono-oxygenase (MMO) enzyme which hydroxylates methane to methanol using molecular O_2 and a reductant (NADH). Indirect evidence for involvement of oxygen in this reaction was first shown by Leadbetter & Foster (1959) who grew *Pseudomonas methanica* in the presence of $^{18}O_2$ and found that if the carbon source was methane, the incorporation of ^{18}O into cell material was 16 times that found when methane was substituted by methanol. This was later

confirmed by Higgins & Quayle (1970) who provided conclusive evidence that during oxidation of methane, whole cells of *Methylomonas methanica* and *Methylomonas methano-oxidans* incorporate ^{18}O into methanol exclusively from molecular oxygen ($^{18}O_2$) but not from water containing ^{18}O.

It is also well established that MMO can exist in either a soluble (sMMO) or membrane-associated particulate (pMMO) form and that the copper to cell biomass ratio in the growth medium is the major factor responsible for determining the nature of the enzyme (Stanley *et al.*, 1983; Prior & Dalton, 1985). At high ratios the particulate form is made, at low ratios the soluble form is made. The two enzyme systems appear to be different. For instance, (a) the carbon conversion efficiency of methane into biomass is 38% higher in cells containing pMMO compared with cells containing sMMO (Leak & Dalton, 1986) and (b) sMMO has the ability to introduce O_2 into a wider range of substrates compared with pMMO (Burrows *et al.*, 1984).

Very little is known about the pMMO. In contrast, three soluble systems have been studied in some detail by Dalton's group. The sMMO from *Methylococcus capsulatus* (Bath) is a three protein component system: component A, the hydroxylase; component B, a regulatory protein and component C, the NADH-driven reductase (Colby & Dalton, 1978; Woodland & Dalton, 1984). These three components were also found in the sMMO from *Methylosinus trichosporium* Ob3b (Stirling & Dalton, 1979; Fox *et al.*, 1989), whereas sMMO from *Methylobacterium* CRL-26 lacks the regulatory protein B (Patel, 1984). The kinetics of the reaction catalysed by this enzyme have been studied by Joergensen (1985), Green & Dalton (1986) and more recently by Dalton *et al.* (1990).

4.1.2.1.2. Oxidation of methanol.

Methanol oxidation in bacteria is catalysed by methanol dehydrogenase (MDH). The first detailed study of the enzymology of methanol oxidation was made by Anthony & Zatman (1964, 1965) in *Pseudomonas* M27. These studies showed that "*in vitro*" MDH activity is independent of nicotinamide nucleotide (NAD^+) but dependent on ammonia or methylamine as activator and on the dyestuff N-methyl-phenazonium methosulphite (phenazine methosulphite) as primary hydrogen acceptor. Five types of this classical MDH, all NAD^+-independent but differing slightly in molecular weight and specificity, have been discovered in a wide range of methylotrophs (Large & Bamforth, 1988). A different MDH (NAD^+-dependent) was, however, isolated by Duine *et al.* (1984) from *Nocardia* sp. 239, a gram-positive organism originally described as *Streptomyces* sp. 239 (Kato *et al.*, 1974). It was found that cell-free extracts of methanol-grown *Nocardia* sp. 239 only show significant dye-linked methanol oxidizing activity when NAD^+ is added to the assay mixture. The MDH was present in a multi-enzyme complex together with NAD-dependent aldehyde and NADH dehydrogenases. Methanol-dependent dye reduction catalysed by this complex required NAD^+. Apart from its requirement for NAD^+, this novel MDH was similar to the classical MDH enzyme in that both contain a prosthetic group called pyrrolo-quinoline-quinone (PQQ) and their oxidation requires ammonia as activator.

The credit for the discovery and evaluation of the role of the PQQ belongs largely to the Dutch scientists Duine and Frank. Using a wide range of chemical and physical techniques (EMR, EPR and ENDOR), they showed that PQQ is a multi-cyclic ring compound with two uncoupled aromatic protons, an inner ring orthoquinone, two nitrogen atoms and one or more carboxyl groups (Duine & Frank, 1981). These descriptions are consistent with those of Salisbury *et al.* (1979) who proposed the name "methoxatin" for the compound. PQQ functions as a hydrogen carrier, undergoing reduction to the quinol form (PQQ $\rightarrow$ PQQH $\rightarrow$

$PQQH_2$), thus transferring the two electrons of the substrate one at a time to the one-electron acceptors of the electron transport chain. Re-oxidation by the electron transport chain occurs at the level of cytochrome C.

Several PQQ containing enzymes (Quinoproteins) have been shown to be involved in bacterial C_1 dissimilation (Duine & Frank, 1990): methylamine dehydrogenase from some Gram-negatives; methylamine oxidase from the Gram-positive *Arthrobacter* P1 and similar enzymes from yeasts; a methanol dehydrogenase from *Clostridium thermo-autotrophicum* and formaldehyde dehydrogenase from methylamine-grown *Hyphomicrobium.*

4.1.2.1.3. Oxidation of formaldehyde.

One of the common biochemical steps amongst methane, methanol and methylamines utilisers is the oxidation of these C_1 substrates to formaldehyde. Formaldehyde is thus a branch-point metabolite in methylotrophic cells, since as mentioned before some methylotrophs assimilate all or a major portion (the rest is further oxidized to CO_2 for energy generation) of the carbon as formaldehyde, whereas others must oxidize all their carbon to CO_2, which is then assimilated *via* the Calvin cycle. Therefore, it is not surprising that almost all methylotrophic cells have been shown to catalyse the oxidation of formaldehyde by means of different enzymes depending on assimilation pathway (Large & Bamforth, 1988). These enzymes are: (1), NAD(P)-linked dehydrogenases; four variants have been reported, but the most studied is formaldehyde dehydrogenase for which the true substrate is *S*-hydroxymethylgluthatione. This enzyme thus requires a second enzyme, *S*-formylgluthatione hydrolase, to convert the product (*S*-formylgluthatione) into formate. Other NAD(P)-linked formaldehyde dehydrogenases do not require gluthatione; (2) dye-linked dehydrogenases: these show less specificity for substrates compared with NAD(P)-linked dehydrogenases and are the most widely distributed amongst methylotrophs (Anthony, 1982); (3) there is considerable

evidence for the presence of a cyclic mechanism for total oxidation of formaldehyde to CO_2 in methylotrophs that uses the hexulose mono-phosphate (HMP) pathway for formaldehyde assimilation (see Large & Bamforth, 1988) and (4) the enzyme system producing formaldehyde from methanol i.e., methanol dehydrogenase has also been suggested (Quayle, 1972, 1980) to play a role in formaldehyde oxidation. Large & Bamforth (1988), however, questioned this possibility since *Pseudomonas* AM1, which contains both MDH and a dye-linked formaldehyde dehydrogenase, can grow on methylamine and oxidize formaldehyde when it has lost MDH by mutation (Heptinstall & Quayle, 1970).

4.1.2.1.4. Oxidation of formate.

Methylotrophic bacteria, without the hexulose-mono-phosphate cycle for carbon assimilation, convert formaldehyde to formate which is further oxidized to CO_2. In most methylotrophs the oxidation of formate to CO_2 is catalysed by an NAD^+-dependent formate dehydrogenase but *Pseudomonas oxaliticus*, an autotrophic methylotroph, has been shown to possess a dye-linked formate dehydrogenase as well as an NAD^+-dependent formate dehydrogenase (Dijkhuizen *et al.*, 1979). The function of the former is mainly to donate electrons to the respiratory chain at the level of $cytochrome_b$ or ubiquinone as it is in *E. coli*.

4.1.2.1.5. Oxidation of methylamines.

A large number of enzyme systems that oxidize methylamines to formaldehyde have been isolated from methylotrophic bacteria. These are mono-oxygenases (Hampton & Zatman, 1973; Boulton *et al.*, 1974), dehydrogenases (Colby & Zatman, 1974) and oxidases (Levering *et al.*, 1981).

4.1.2.1.6. Oxidation of carbon monoxide.

Bacteria that grow on carbon monoxide (carboxydotrophs) as a sole source of energy and carbon do so by oxidizing CO to CO_2 *via* a dye-linked CO dehydrogenase (Meyer, 1985). The CO_2 formed is subsequently assimilated in the Calvin cycle. CO dehydrogenase has been found to be a molybdo-iron-sulphur flavoprotein containing bactopterin in its molybdenum cofactor (Krüger & Meyer, 1987).

4.1.2.2. Assimilatory pathways.

There are three main pathways by which carbon is assimilated in methylotrophic bacteria, namely the Calvin cycle, the Hexulose mono-phosphate pathway and the Serine pathway.

Early work suggested that a particular C_1-utilizing bacterium utilises only one of these pathways (Quayle, 1972). However, it is now clear that under certain growth conditions some organisms may change the assimilatory pathway used for C_1 assimilation. The main findings supporting this statement can be summarized as follows: (a) Taylor (1977) demonstrated that *Methylococcus capsulatus* (Bath) which has a highly active HMP cycle, also contains ribulose-bis-phosphate carboxylase from the Calvin cycle during growth on methane; (b) *Pseudomonas gazotropha* uses the Calvin cycle during growth on carbon monoxide whereas the serine pathway is used when assimilating methanol (Romanova & Nozhevnikova, 1977) and (c) low to moderate activities of hydroxypyruvate reductase, an enzyme normally associated with the serine pathway, have been detected in extracts of (i) a number of organisms known to use the HMP pathway as their major pathway for C_1 assimilation (Lawrence & Quayle, 1970; Strom *et al.*, 1974) and (ii) *Paracoccus denitrificans* which usually uses the Calvin cycle as its main pathway for C_1 assimilation (Cox & Quayle, 1975; Bamforth & Quayle, 1977). These data led to

the conclusion that the detection of certain enzyme activities in bacterial extracts is not always conclusive evidence for the utilization of the corresponding metabolic pathway as the main pathway for C_1 assimilation (Bamforth & Quayle, 1977).

4.1.2.2.1. The Calvin cycle.

This cycle, which is also known as the ribulose-bis-phosphate pathway or the reductive pentose phosphate cycle, was the first sequence of reactions found to effect the net synthesis of a C_3 compound from a C_1 compound (Bassham *et al.*, 1954). The overall metabolic sequence (Fig. 9) can be divided into three steps:

(a)- Fixation, which involves the formation of a C_3 compound "3-glycerate 3-phosphate" as a result of a reaction between 3 molecules of CO_2 and 3 molecules of ribulose-bis-phosphate catalysed by ribulose-bis-phosphate carboxylase.

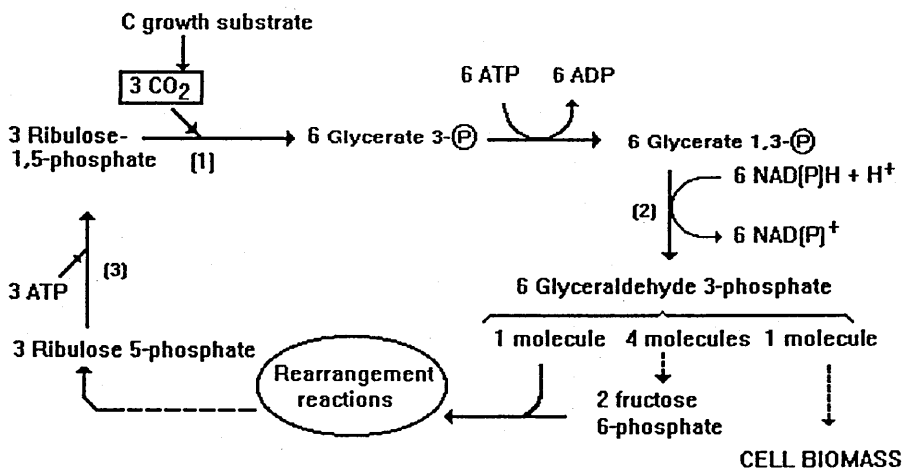
(b)- Reduction, in which 6 molecules of glycerate 1,3-phosphate, which arise from the phosphorylation of glycerate 3-phosphate by a specific kinase, are reduced to 6 glyceraldehyde 3-phosphate by the action of glyceraldehyde 3-phosphate dehydrogenase. One molecule of these is used for biosynthesis, and

(c)- Rearrangement, where 4 molecules of the remaining glyceraldehyde 3-phosphate enter the gluconeogenic pathway to yield two molecules of fructose 6-phosphate *via* triose-p-isomerase, fructose-bis-phosphate aldolase, and fructose bis-phosphatase. These two molecules of fructose 6-phosphate then enter (with the remaining molecule of 3-phosphoglyceraldehyde) a series of reactions to give 3 molecules of ribulose 5-phosphate. The latter are then phosphorylated by the action of phosphoribulokinase to yield 3 molecules of ribulose-bis-phosphate, thereby completing the cycle.

Energy consumption in this cycle is very high in terms of both ATP and reducing equivalents as shown in the overall stoicheiometry of the cycle, which can be written as follows: 3 RuBP + 3 CO_2 + 9 ATP + 6 NAD(P)H + 6 H^+ $\rightarrow$ Glyceraldehyde 3-phosphate + 3 ribulose bis-phosphate + 6 $NAD(P)^+$ + 9 ADP + 8

Pi. This rather high demand for energy, as compared to the other 2 assimilatory pathways, perhaps explains the rarity of this process amongst heterotrophic organisms.

Figure 9. The Calvin (ribulose 1,5-bisphosphate) cycle of CO_2 fixation.*



Key enzymes: (1), Ribulose bisphosphate carboxylase; (2), Glyceraldehyde 3-phosphate dehydrogenase and (3), Phosphoribulokinase.

* Adapted from Large (1983).

4.1.2.2.2. The Serine pathway.

Up to 1959, it was believed that all methylotrophs assimilate their carbon *via* the Calvin cycle, but in that year the occurrence of the serine pathway was demonstrated (Kaneda & Roxburgh, $1959_{a,b}$). Incorporation, after short incubation periods, of ^{14}C from [^{14}C]methanol by methanol-grown *Pseudomonas* PRL. W4 (Kaneda & Roxburgh, 1959_a) and by methanol- and formate-grown *Pseudomonas* AM1 (Large *et al.*, 1961) demonstrated the basis of this pathway. These studies showed that: (a) the first stable products of [^{14}C]methanol fixation were serine (about 50% of total carbon fixed) and C_4 dicarboxylic acids such as malate and aspartate; (b) the radioactivity incorporated from [^{14}C]methanol into compounds other than serine, malate and aspartate appeared mainly in glycine at early times of

incubation and in some phosphorylated compounds which appeared later; and (c) the specific radioactivity of cell material obtained from methanol-grown *Pseudomonas* AM1 in air was decreased by 50% on bubbling air-CO_2 (99:1, v/v) mixture through the culture medium. This was interpreted as suggesting that at least 50% of the carbon incorporated must have passed through the stage of carbon dioxide or a compound in ready equilibrium with it (Large *et al.*, 1961).

These findings indicate that, unlike the Calvin cycle, only 30 to 50% of the cell carbon material is derived from CO_2 in this pathway (Large *et al.*, 1961), the rest of carbon is incorporated at the level of formaldehyde. CO_2 fixation was later found to be catalysed by phosphoenolpyruvate carboxylase (PEPc) (Large *et al.*, 1962) .

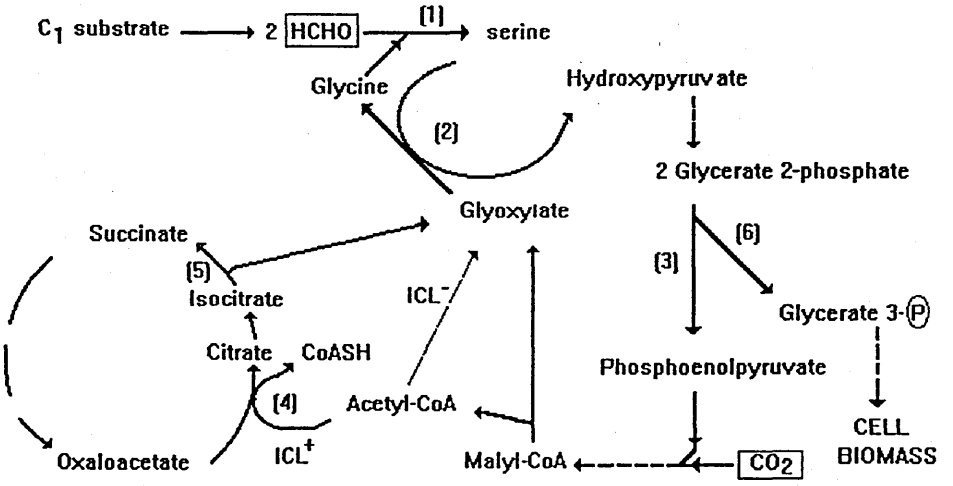
Like the Calvin cycle, the serine pathway also can be divided into three phases (Fig. 10):

(a)- Fixation, in which formaldehyde enters a series of reactions catalysed by serine hydroxymethyltransferase, serine glyoxylate aminotransferase, hydroxypyruvate reductase and glycerate kinase. This leads to the formation of glycerate 2-phosphate which is then converted to PEP *via* PEP hydratase. The PEP then enters (with CO_2) another series of reactions to yield a C_2 compound "Acetyl-coenzyme A".

(b)- Conversion of Acetyl- coenzyme A into glycine (the C_1 acceptor) *via* glyoxylate. Two variants of this phase have been shown in methylotrophic bacteria. The so-called ICL^+ variant where acetyl-coenzyme A is converted first into isocitrate (by some enzymes of the TCA cycle) which is then cleaved to yield glyoxylate and succinate. An ICL^- variant operates in certain methylotrophs such as *Pseudomonas* AM1 but its definite route is still unresolved (Large & Bamforth, 1988).

(c)- The phase where glycine and another molecule of formaldehyde are converted, *via* a series of reactions similar to those of phase (a), to glycerate 3-phosphate which serves as a precursor for cell material.

Figure 10. The Serine pathway of carbon assimilation (ICL^+ variant).*



Key enzymes: (1) Serine hydroxymethyltransferase, (2) Serine glyoxylate aminotransferase, (3) Phosphoenolpyruvate hydratase, (4) Citrate synthase, (5) Isocitrate lyase and (6) Phosphoglycerate mutase.

* Adapted from Large & Bamforth (1988).

The overall stoichiometry of the serine pathway can be summarised as follows: 2 HCHO + CO_2 + 3 ATP + 2 NAD(P)H + 2 H^+ → Glycerate 3-phosphate + 2 $NAD(P)^+$ + 2 H^+ + 3 ADP + 2 Pi + H_2O.

4.1.2.2.3. The Hexulose mono-phosphate pathway (HMP).

This pathway, which is also known as ribulose mono-phosphate pathway (RuMP), was first proposed by Quayle and co-workers in *Pseudomonas* (*Methylomonas*) *methanica*. The studies of Johnson and Quayle (1965) showed that [^{14}C]methane and [^{14}C]methanol are rapidly assimilated by methane- and methanol-grown cell suspensions mainly into sugar phosphates, whereas [^{14}C]bicarbonate is incorporated mainly into aspartate and malate. These findings together with the failure to detect any activities for carboxydismutase, a key enzyme of autotrophic metabolism, in

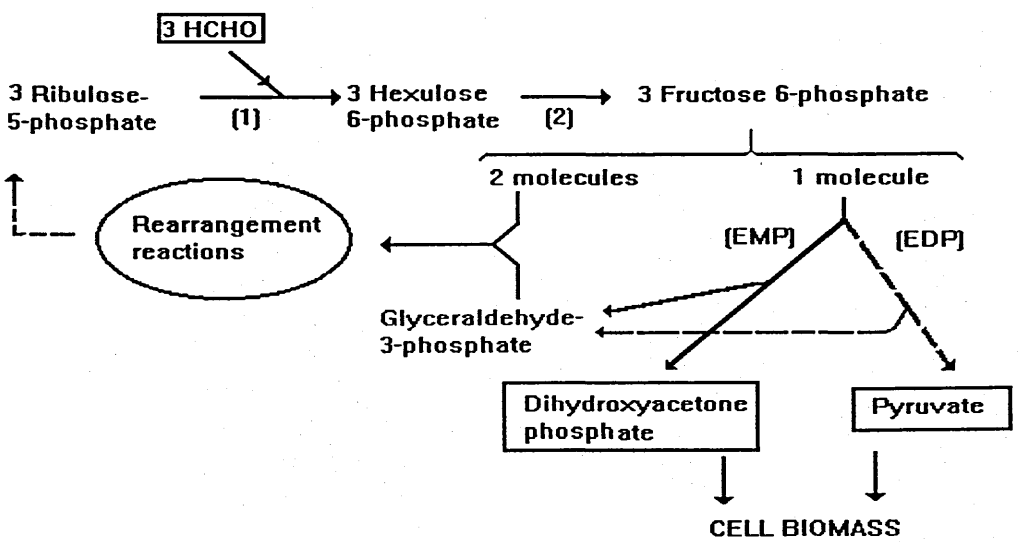
cell-free extracts of *Ps. methanica* led the authors to conclude that an autotrophic metabolism involving the ribulose bis-phosphate cycle cannot be operating. Instead they suggested that C_1 units might be assimilated at reduction level(s) between methanol and formate by an unknown pathway. Subsequent work by Kemp & Quayle (1966) showed that incubation of cell-free extracts of methane- or methanol-grown *Ps. methanica* with [^{14}C]formaldehyde and D-ribose 5-phosphate leads to incorporation of ^{14}C into a non-volatile product, which has the chromatographic properties of a phosphorylated compound. Treatment of this compound with a phosphatase revealed the presence of two free sugars "fructose and allulose". From this a variant of the ribulose-bis-phosphate cycle, involving the condensation of formaldehyde (as the C_1 unit) with ribose 5-phosphate to give allulose 6-phosphate, was suggested as an alternative pathway for C_1 assimilation in *Pseudomonas methanica.* In support of this statement (Kemp & Quayle, 1967) demonstrated that whereas ^{14}C from [^{14}C]formate was fixed into serine and malate, radioactivity from [^{14}C]formaldehyde appeared mainly in sugar phosphates such as glucose and allulose. Activities of the enzyme system catalysing the condensation reaction in this new pathway were detected later by Lawrence *et al.* (1970) in extracts of *Methylococcus capsulatus.* From this it was concluded that during growth of *Methylococcus capsulatus* on methane, as with *Ps. methanica,* cell constituents are made by the Hexulose mono-phosphate pathway (HMP) of formaldehyde fixation. The reactions of this new pathway are analogous to those of the Calvin cycle. The intermediates of both pathways are carbohydrates, compared to the serine pathway where the intermediates are mainly amino acids and carboxylic acids. However, the HMP pathway is more economical energetically than the Calvin cycle since it does not require the reductive step (the second step in the Calvin cycle, Fig. 9) because it is formaldehyde, which is more reduced than CO_2, that is oxidized.

This pathway also is divided into three steps (Fig. 11). In step A, the reaction of formaldehyde with ribulose 5-phosphate, catalysed by hexulose

phosphate synthase, forms D-erythro-L-glycero-3-hexulose 6-phosphate, which is then isomerized to fructose 6-phosphate by hexulose phosphate isomerase. Step B involves cleavage of one molecule of the resulting fructose 6-phosphate into two C_3 compounds. One of these is glyceraldehyde 3-phosphate, which forms the starting material for step (C) whereas the nature of the second compound, which will eventually be used for biosynthesis, depends on which variant (Emden-Meyerhof Pathway, EMP or Entner-Doudoroff Pathway, EDP) of the cycle operates in a given organism. Step C involves the rearrangement of the triose- (glyceraldehyde 3-phosphate) and the remaining hexose-phosphates to regenerate the ribulose 5-phosphate (the formaldehyde acceptor).

Thus, in the overall process of the HMP pathway, 3 C_1 molecules (as formaldehyde) condense with 3 C_5 sugar phosphates to give three C_6 sugars, one of which is cleaved to provide a C_3 which is used for biosynthesis. The remaining 15 carbon units are then rearranged to regenerate the original three C_5 sugars (acceptors of HCHO) molecules.

Figure 11. The Hexulose mono-phosphate pathway of formaldehyde fixation.*



Key enzymes: (1), Hexulose phosphate synthase; and (2), Hexulose phosphate isomerase.

* Adapted from Large (1983).

4.1.3. Utilization of C_1 compounds by N_2-fixing bacteria.

The ability of bacteria to use C_1 compounds and atmospheric nitrogen as carbon and nitrogen sources for growth, respectively, has been known for a long time. Indirect evidence for nitrogen fixation by methane-oxidizing bacteria was first obtained in the 1930's (Schollenberger, 1930; Harper, 1939). In an investigation to determine the effect of leaking natural-gas upon the soil Schollenberger (1930) noticed that soil exposed for a prolonged period of time to natural-gas escaping from leaking line pipes had considerably higher nitrogen content than unexposed soil. Harper (1939) specifically studied the effect of natural-gas leaks on the accumulation of nitrogen and organic matter in soil. He found that organic nitrogen of ten soils exposed to natural-gas leaks averaged 0.26%, as compared to only 0.07% for control soils. Organic matter was also higher in exposed soils. Although neither of these researchers isolated or identified C_1-oxidizing bacteria from the soil samples studied, since methane had been shown to be the main component of natural-gas (Burrell & Oberfell, 1915), these observations were interpreted as suggesting that methane-oxidizing bacteria were responsible for fixing extra nitrogen.

Partial support for this interpretation was provided by Davis (1952) who isolated bacteria capable of utilizing methane and other gaseous hydrocarbons from soil samples taken from a natural-gas seep "paraffin dirt" bed. Analysis showed that the soil used had a high organic nitrogen content (1.2%). No attempt, however, was made to test the ability of this newly isolated bacteria (*Pseudomonas methanitrificans*) to fix nitrogen. Further support was provided by the studies of Davis *et al.* (1964) and then Coty (1967). The latter while studying the effect of methane on nitrogen content of soil recorded similar effects to those caused by natural-gas (Schollenberger, 1930; Harper, 1939). Like Davis *et al.* (1964) he also isolated a methane-oxidizing *Pseudomonas* strain from his soil samples. In addition, by using $^{15}N_2$ enrichment, he was able to demonstrate the ability of this new isolate

to fix nitrogen. Whittenbury *et al.*(1970) confirmed these results by isolating another methane-oxidizing strain "*Methylosinus trichosporium*" from Coty's culture. By employing the acetylene-ethylene assay, they showed that this new strain was able to reduce 25% of the acetylene present during an incubation period of 7 to 14 days. This was soon followed by the studies of de Bont & Mulder (1974) and de Bont (1976) which showed that both methane-oxidizing *Methylosinus*-type and *Methylomonas*-type strains have the ability to fix nitrogen.

Many other C_1 utilizing bacteria, possessing the capacity for nitrogen fixation, have now been identified. These include *Methylobacter* spp., *Methylococcus* spp., *Methylocystis* spp., *Rhodopseudomonas* spp., *Thiobacillus* spp., and *Bacillus* spp. It is not surprising, however, that these genera have the ability to use C_1 compounds, because of their widespread occurrence in habitats where these compounds are abundant (see general introduction).

Actinomycetes are another group of bacteria that occur in a wide variety of natural and man-made habitats, growing on a vast range of substrates within them. Nutritionally most of the actinomycetes are saprophytes, the rest form either parasitic or symbiotic associations with plants or animals (Williams *et al.*, 1984).

Few free-living actinomycetes have been claimed to fix nitrogen. However, the literature is rather conflicting with regard to the validity of these claims. Smyk & Ettlinger (1963) reported that 7 *Arthrobacter* strains isolated from the surface of karstic rocks were able to fix nitrogen. In contrast, Mulder & Brotonegoro (1974) pointed out that nitrogen fixation, if it does occur, in the genus *Arthrobacter* must be very rare as they failed to detect any nitrogen fixation in more than 100 strains tested in their laboratory. Nitrogen-fixing members of the genus *Mycobacterium* were first isolated from Russian turf-podzol soils by Fedorov & Kalininskaya (1961) and then L'vov (1963). Biggins & Postagate (1969) re-examined *Mycobacterium flavum* 301 and confirmed its ability to fix nitrogen. Few years later, however, this species and other related organisms were re-classified as

Xanthobacter spp. (Wiegel *et al.*, 1978; Malik & Claus, 1979). Earlier claims that *Nocardia calcarea* was able to fix nitrogen (Metcalfe & Brown, 1957) have also been repudiated (Hill & Postage, 1969). Moreover, in a more recent survey Pearson *et al.* (1982) failed to detect nitrogenase activity in a number of actinomycetous species belonging to the genera *Nocardia*, *Oerskovia*, *Rhodococcus*, and *Streptomyces*.

With respect to C_1-utilization, a number of species belonging to three actinomycetous genera have been shown to utilize C_1 compounds as sole carbon source for growth. These are *Arthrobacter* spp. (Loginova & Trotsenko, 1975; Colby & Zatman, 1975; Levering *et al.*, 1981), *Mycobacterium vaccae* 10 (Hanson, 1980) and *Nocardia* sp 239 (Kato *et al.*, 1974, 1977; Hazeu *et al.*, 1983; Duine *et al.*, 1984). The most extensively studied of these is the methanol-oxidizing *Nocardia* sp. 239 which was first isolated by Kato *et al.* (1974). They identified the organism as *Streptomyces* sp. No.239, but a few years later, based on acid fast staining, morphology of the cells and the composition of cell wall studies of de Vries, Hazeu *et al.*, (1983) concluded that the organism probably belongs to the genus *Nocardia* and thus proposed a new name "*Nocardia* sp. No239". Enzymological studies with cell-extracts of the organism growing on methanol detected low levels of both hexulose phosphate synthase and hydroxypyruvate reductase (Kato *et al.* (1977). This was interpreted as suggesting that both the HMP pathway and the serine pathway were operative in this organism. However, later studies by Hazeu *et al.* (1983) who unlike Kato *et al.* (1977) used continuous culture rather than batch culture, detected higher levels of hexulose phosphate synthase in methanol-grown cells. They concluded that methanol is assimilated *via* the HMP pathway. They also pointed out that the methanol-oxidizing enzyme in *Nocardia* sp. No239 is different from the one encountered up to then in methanol-oxidizing bacteria. Subsequent confirmation of this statement was provided by Duine *et al.* (1984) (see page 115).

Other actinomycetes that can oxidise C_1-compounds but lack the ability to fix nitrogen are: *Brevibacterium* sp. 24 (Hanson, 1980) *Corynebacterium* sp. (Mimura *et al.*, 1978) and *Micrococcus denitrificans* (Cox & Quayle, 1975).

In contrast to free-living actinomycetes, the nitrogen-fixing ability of some actinomycetes living as microsymbionts in root nodules of actinorhizal plants is well established. These species are all placed under the genus *Frankia* (see general introduction). In contrast to early suggestions (Becking, 1970) that *Frankia* could not exhibit extra-nodular growth, it is now almost certain that like the majority of actinomycetes *Frankia* also is able to grow saprophytically in soil. For instance, Van Dijk (1979) concluded from his studies on infection rates of potential host plants that the presence of a suitable host was the prime factor but that extranodular growth or long-term survival of the bacterium could not be ruled out. In addition, the capacity of at least some *Frankia* to grow, fix nitrogen and sporulate in simple media in *vitro* is further indirect evidence that *Frankia* can grow in soil. Furthermore, in a recent study Smolander & Sarsa (1990) confirmed earlier reports (e.g., Rodriguez-Barrueco, 1968; Van dijk, 1984; Weber, 1986; Arveby & Huss-Danell, 1988; Smolander & Sundman, 1989) that infective *Frankia* endophytes can be obtained from soils that either were never known to support host plants or had been free from these trees for many years.

As described in the general introduction methane is widely distributed in habitats common for some actinorhizal species. We also have seen that methanol is formed in nature by either oxidation of methane or by release of methoxy groups from lignin and pectin, which are quantitatively important components of plant biomass. Data in support of the long-held belief that actinomycetes decompose lignin and cellulose in soil has been obtained recently. Streptomycetes degrade intact cell walls of phloem from Douglas fir (Sutherland *et al.*, 1979) and the lignocelluloses of grass, softwoods and hardwoods (Anti & Crawford, 1981). Moreover, *Nocardia* spp. from soil released $^{14}CO_2$ from labelled coniferyl alcohol,

the main precursor of lignin biosynthesis, and from plant lignins (Trojanowski *et al.*, 1977). Degradation of lignin-related compounds by *Nocardia* and *Rhodococcus* strains has also been shown (Eggeling & Sahm, 1980, 1981; Rast *et al.*, 1980).

Although the ability of *Frankia* to degrade lignin has not been reported, Donnelly *et al.* (1981) have shown that the breakdown of lignin monomers and related compounds by *Arthrobacter* sp. strain TMP gives rise to methanol, which is not further metabolized by this organism and is thus available for metabolism by other organisms present in that environment. In addition, early findings that the *Alnus glutinosa* root nodules contain active pectolitic enzymes capable of degrading the pectic capsule surrounding the endophyte (Schouten & Lalonde, cited by Lalonde, 1977), suggest that *Frankia* may be able to utilise for growth *in vivo* methanol produced by the degradation of cell wall pectin during the infection process. Thus it was our aim in this part of the project to examine the possibility that like some other actinomycetes *Frankia* also might have the ability to utilize C_1 compounds such as methane, methanol and formate as sole carbon sources for growth.

4.2. MATERIALS AND METHODS

In this section, all aseptic techniques, maintenance of cultures, preparation of inoculum, quantification of *Frankia* biomass and statistical analysis procedures were as described in Materials and Methods of Chapter 3.

4.2.1. Growth on C_1 compounds.

A number of *Frankia* strains were examined for their ability to utilise methane, methanol or formate as sole carbon source. The strains tested, all of which were routinely maintained previously on BuCT, were harvested and homogenised as before and then dispensed into 250-ml conical flasks containing 100 ml. of Bu-C medium. The flasks, plugged with cotton wool and closed with aluminium foil, were incubated in the dark at 27 °C, under non-shaking conditions.

Two weeks later, starved cultures were filtered and the resulting mycelia resuspended in Bu-C medium, homogenised as before and equal aliquots of inoculum homogenates were used for inoculating glass-tubes (2 cm x 15 cm) containing 10 ml. of the desired growth medium.

In cultures involving growth on methanol or formate as sole carbon source, propionate was omitted from Bu medium and methanol, 5 $ml.L^{-1}$ (added by filter sterilisation) or sodium formate, 0.5 $g.L^{-1}$, were substituted. After inoculation, the culture-tubes were covered with aluminium caps, which were sealed to the tubes with "Nescofilm", and incubated in the dark at 27 °C, under non-shaking conditions.

In cultures designed to test the ability of *Frankia* strains to grow on methane as carbon source, the inoculum homogenates were transferred to tubes containing 10 ml. of Bu-C medium. The tubes, plugged with cotton wool, were randomised within 1.4 l-capacity glass jars having lids fitted with rubber stoppers to facilitate adjustment of the gas mixture (5% methane + 95% air) within the incubation jars. This was done by withdrawing 5% air from each incubation jar and replacing it with

an equivalent volume of methane, using a syringe. The jars were incubated in the dark at 27 °C, under non-shaking conditions and the gas mixtures were changed and adjusted in the same manner twice a week.

4.2.2. Metabolism of C_1 compounds.

4.2.2.1. Media and growth conditions.

The strains tested, all of which were routinely maintained previously on BuCT, were harvested, homogenised and then starved on Bu-C as described before. Two weeks later, starved cultures were filtered and the resulting mycelia resuspended in Bu-C medium, homogenised as before and the resulting suspensions used for inoculating 100-ml conical flasks containing 50 ml. of Bu medium, but with 0.5 % (v/v) methanol (added by filter sterilization) substituted for propionate as the sole carbon source. The flasks, plugged with cotton wool and closed with aluminium foil, were incubated in the dark at 27 °C, under non-shaking conditions.

4.2.2.2. Incorporation of ^{14}C-methanol into amino acids by methanol-grown *Frankia*.

Incorporation of ^{14}C-methanol and preparation of bacterial cell-free extracts:

After 7 weeks of growth under the conditions described in 4.2.2.1., the cells were reactivated by transferring the mycelium into one flask containing 100 ml of fresh growth medium. Reactivated cells were reincubated under the same conditions as before.

A week later, 2.5 ml of ^{14}C-methanol (containing 25 µCi of ^{14}C) were added to the flask. The cells were incubated at 30 °C for 1 hour before harvesting on 0.2 µm membrane filter, using a Millipore unit. The cells were washed several times to remove exogenous radioactivity before transfer in 100% ethanol into a homogeniser, after which they were homogenised and heated at 75 °C, for 5 min. The ethanolic suspensions of killed cells were diluted to 80% ethanol with distilled water, shaken

for 5 min. at 75 °C and harvested again on 0.2 µm membrane as before. The filtrate was reduced to dryness in a rotary evaporator, dried under nitrogen and redissolved in water prior to derivatization and amino acid analysis by high-performance liquid chromatography (HPLC).

Derivatization:

Extracts and standards were dissolved in 200 ul of distilled water in a glass V-vial and mixed with 50 µl 1 M borate KOH, pH 6.3, and 200 µl 15 mM 9-fluorenylmethoxycarbonyl chloride, FMOC-cl (Sigma, Poole, Dorset, UK). The reaction mixtures were reacted for 1 min at room temperature then were partitioned 3 times against 500 µl pentane to remove excess FMOC after which aliquots of the residual aqueous phase were analysed by HPLC.

HPLC:

Reversed-phase HPLC of derivatized standards and extracts was carried out on a 250 x 5 mm i.d column packed with 5 µm ODS Hypersil support (Shandon, Runcorn, Cheshire, UK). Solvents (A: was either 0.5 M acetic acid adjusted to pH 2.8 or 0.1 M acetic acid, containing 0.1% triethylamine, adjusted to pH 4.2; and B: was methanol) were delivered at a flow rate of 1 ml min-1 by a Spectra Physics 8100 liquid chromatography (San Jose, CA, USA). The HPLC eluate was directed to a radioactivity monitor (Reeve analytical) fitted with a 500 µl homogeneous flow cell and fractions (30 sec.) collected in a Gilson FC 203 fraction collector. The detector was a Perkin-Elmer LS-3 spectrophotofluorimeter (Beaconsfield, Bucks, UK) fitted with a 12 µl flow cell (excitation 250 nm, emission 315 nm).

4.2.2.3. Incorporation of ^{14}C-formaldehyde into sugar phosphates by methanol-grown *Frankia.*

Standard sugars: The sugar phosphates (D-ribose, ribose 5-phosphate, D-fructose, fructose 6-p, D-xylulose and xylulose 5-p) were purchased from Sigma.

Solvent and spray reagents: These were as those of Waring & Ziporin (1964):

The solvent used was a water-poor phase from mixture of: 6g of toluene-4-sulphonic acid dissolved in 180 ml *tert*-amyl alcohol and 90 ml distilled water.

The sprays were: spray (A), which consisted of 0.5 g phenylenediamine + 1.2 g stanous chloride in 80 ml ethanol and 20 ml glacial acetic acid and spray (B), which consisted of 5 ml of 60 % perchloric acid, 10 ml of 1N HCl, 25 ml of 4 % ammonium molybdate and 60 ml distilled water.

Plates: Pre-coated 20 x 20 cm TLC plates cellulose (without fluorescence indicator) were purchased from Merck.

Preparation of cell-free extracts:

After 7 weeks of growth under the conditions described in 4.2.2.1, the cells were reactivated as before. A week later the cells were harvested on 0.2 µm membrane using a Millipore unit. The resulting mycelium was washed thoroughly with 50 mM Na-phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$, resuspended in buffer - 1 mg of dithiothreitol was added to prevent oxidation - and disintegrated by sonication at full power for 3 min. The crude extract was transferred to a microconcentrator (Centricon™ 10) and centrifuged at 7,000 x g for 30 min. at °C. The supernatant was kept on ice until used as crude cell-free extract.

Incubation of cell-free extracts and sugar phosphates with ^{14}C-formaldehyde:

Incubation of cell-free extracts and sugar phosphates with ^{14}C-formaldehyde was carried out as described by Lawrence *et al.* (1970). The complete reaction mixture contained: 20 µmol of Na-phosphate buffer, pH 7.0; 2 µmol of $MgCl_2$; 2 µmol of

Ribose 5-P or Xylulose 5-P; 2 µmol of ^{14}C-formaldehyde, containing 0.05 µCi of ^{14}C; 150 µl of cell-free extract (containing 0.1 mg protein) and buffer in a total volume of 0.4 ml. Either the sugars or the extracts were omitted from the controls. The reactions were started by the addition of extracts and proceeded for 5 min. at 30 °C before stopping for determination of radioactivity incorporation as described below.

Measurements of radioactivity incorporation and identification of labelled compounds:

The incorporation of radioactivity onto sugar phosphates after incubation with radioactive substrate was determined by two different methods:

(1) Precipitation of sugar phosphates (Lawrence *et al.*, 1970). The reaction mixtures were stopped with 1.5 ml ethanol and 0.1 ml of 5 % (w/v) barium acetate was added in order to precipitate sugar phosphates. The mixtures were filtered through circles of glass-fibre paper (Whatmann) and washed with ethanol. Each glass-fibre disc containing the precipitate was sucked dry, under vacuum, before transfer to scintillation vials for radioactivity assay as before.

(2) Chromatography (Kemp & Quayle, 1966). The reaction mixtures were stopped with 1.6 ml methanol, evaporated to dryness with N_2 and the residues taken-up either in 80 % methanol or distilled water. One mg of phosphatase acid was added to each of the extracts dissolved in water. In order to dephosphorylate the phosphorylated compounds by the action of phosphatase, these extracts were incubated at 30 °C for 3 hours prior to chromatography.

Aliquots of both extracts (methanolic and water-soluble extracts) were chromatographed one-dimensionally alongside authentic samples of phosphorylated and dephosphorylated sugar standards on the same plate in the solvent system described before. The spots were applied, along a line approximately 2.5 cm above the cellulose bottom edge, with a glass capillary and were permitted to dry between applications to minimize origin size as much as possible. The solvent system was allowed a 1 hour period to saturate the atmosphere inside the glass-chromatojar, after

which the plates were positioned in the chromatojar for ascending chromatography. Seven to eight hours were required for the solvent front to move 10-10.5 cm after which the plates were removed from the chromatojar and air dried at room temperature, usually overnight.

After drying was complete the cellulose was divided into 2 cm wide segments and the segments through which the extract spots were run were divided into rectangles (2 cm wide x 0.5 cm high). Each rectangle was scraped into a scintillation vial and 0.2 ml of methanol added per vial. An hour later, the samples were assayed for radioactivity as before.

The chromatoplates, still containing the segments through which the sugar standards ran, were sprayed with sprays A and B, which gave characteristic colour responses, as follows: Spray A was applied first after which the plates were heated for 5 min. at 100 - 110 °C and spots marked as visualised. After cooling, spray B was applied and the plates were dried with a hair drier before heating for 10 min. at 100 - 110 °C. The spots were marked as visualised for R_F measurements.

4.3. RESULTS.

4.3.1. Growth on C_1 compounds.

Data obtained from an experiment designed to test the ability of a number of *Frankia* strains to utilize methane, methanol or formate as sole sources of carbon are summarised in Table 13 and Figure 12.

Table 13 shows that six of eight *Frankia* strains tested used methanol and all three strains tested utilized formate as sole source for growth, but only two of eight strains tested (ArI4 and 3.1.5P) showed slight growth with methane.

Table 13. Growth of *Frankia* with methane, methanol or formate as carbon source.

Strains	Growth yield (µg protein / 10-ml culture)			
	Control	Methane (1), 5 % (v / v)	Methanol, (5ml/L)	Formate, (0.5g/L)
ArI3	15.8±1.78	14.3±1.15	14.6±0.94	
ArI4	11.1±0.92	13.0±0.51 *	16.0±1.36 **	
ArI4	12.0 ±0.71			16.2±0.46**
1.2.5Q	10.5±1.19	10.9±2.83	11.6±1.36	
1.1.5F	7.3±0.89	8.0±1.60	11.2±1.00 **	
1.1.7F	9.6±1.40	11.3±1.09	13.7±0.90 **	
1.1.8Bu	8.6±0.94	10.4±1.41	11.5±1.26 **	
3.1.5P	9.1±0.71	11.5±0.66 *	14.1±0.28 ***	
3.1.5P	10.6±1.21			14.7±0.67**
3.1.10P	8.0±0.66	8.0±1.14	13.8±0.96 ***	
3.1.10P	9.1±1.08			14.9±1.17 ***

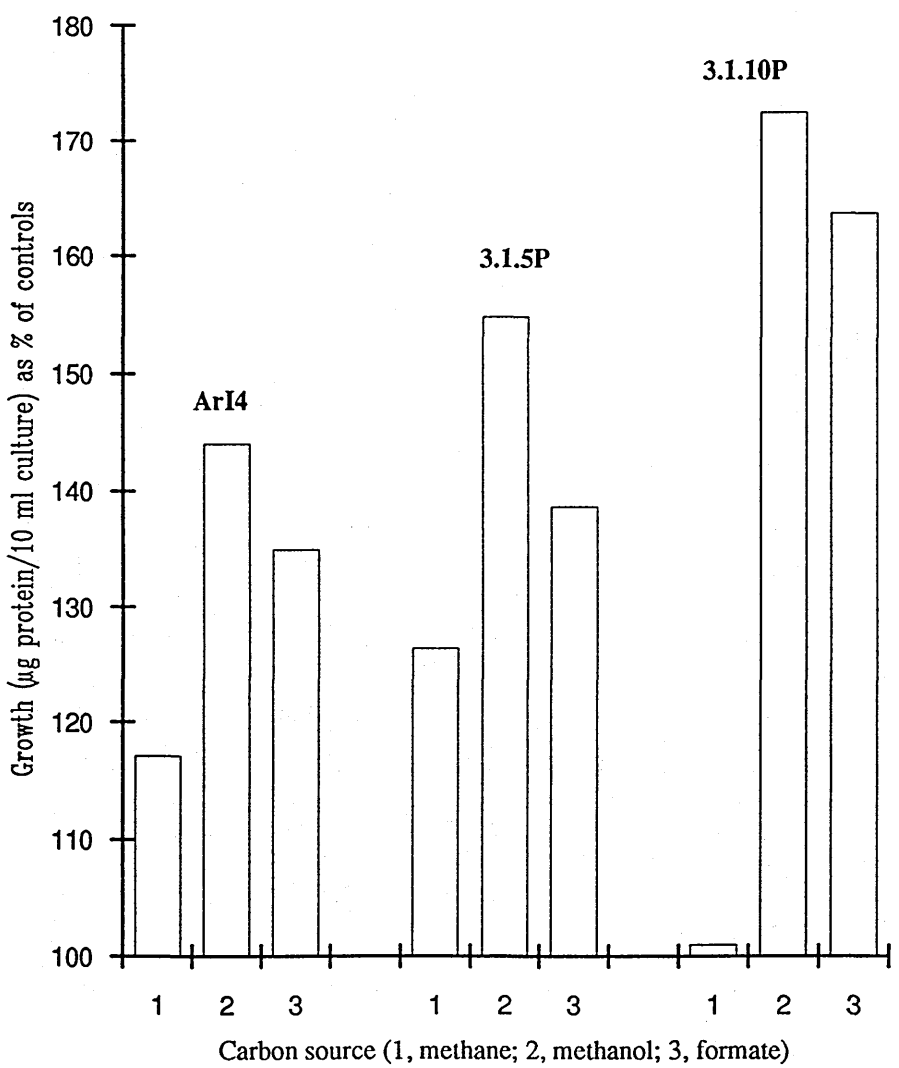
The growth was assessed 7 weeks after inoculation and data are mean ± SE, (n=3).

(1) The atmosphere within the incubation jars was (5 % methane + 95 % air).

*, ** and ***: Data are significantly different from the controls at $P \leq 0.1$, $P \leq 0.05$ and $P \leq 0.01$, respectively.

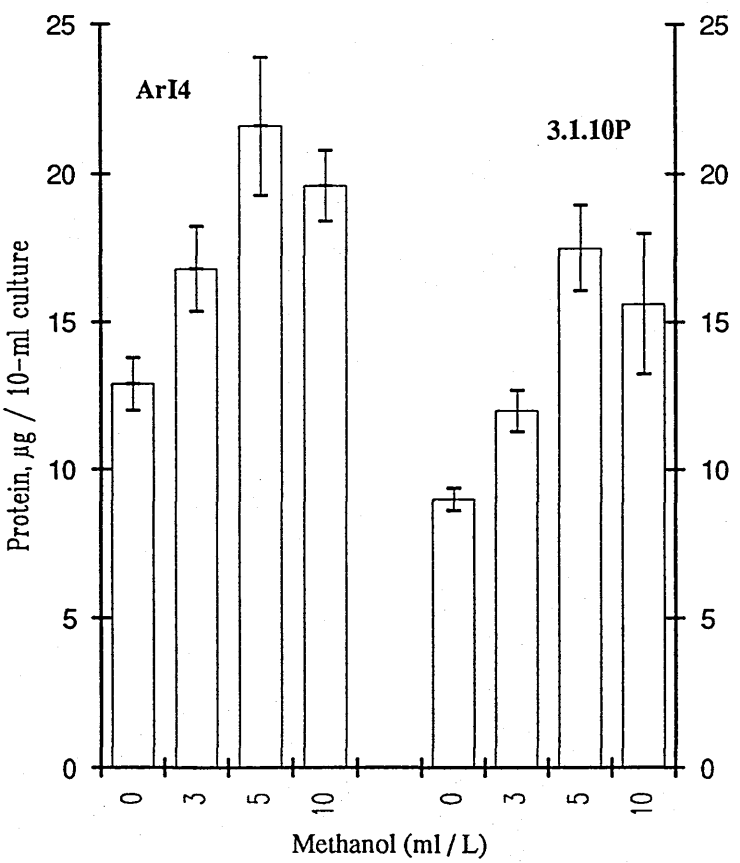
Figure 12 shows that addition of methanol to the growth medium increased the growth yield (total protein) of ArI4, 3.1.5P and 3.1.10P by 45, 55 and 70%, respectively. Addition of formate also resulted in relatively high increases in yield but these increases were smaller that those obtained with methanol with all strains tested. Of the strains tested 3.1.10P showed the best growth both with methanol and formate. However, this strain showed no growth with methane, which improved the growth yield of ArI4 and 3.1.5P by 17 and 26.5%, respectively.

Figure 12. Percentage increase in growth of *Frankia* cultures grown on C_1 compounds compared with cultures grown on C - free medium (controls). Data were calculated from Table 13.



In a second experiment, the effect of methanol concentration in the growth medium on *Frankia* growth (total protein yield) was examined. The results of this experiment are summarised in Fig. 13. The histogram shows that total protein yields of ArI4 and 3.1.10P were increased by 65 and 95%, respectively, as the methanol concentration was increased from 0 to 0.5% (v/v). Further increase of the methanol concentration to 1% did not result in any additional significant increase in protein yield of either strain.

Figure 13. Effect of methanol concentration in the growth medium on growth of *Frankia* strains cultivated for 45 days.

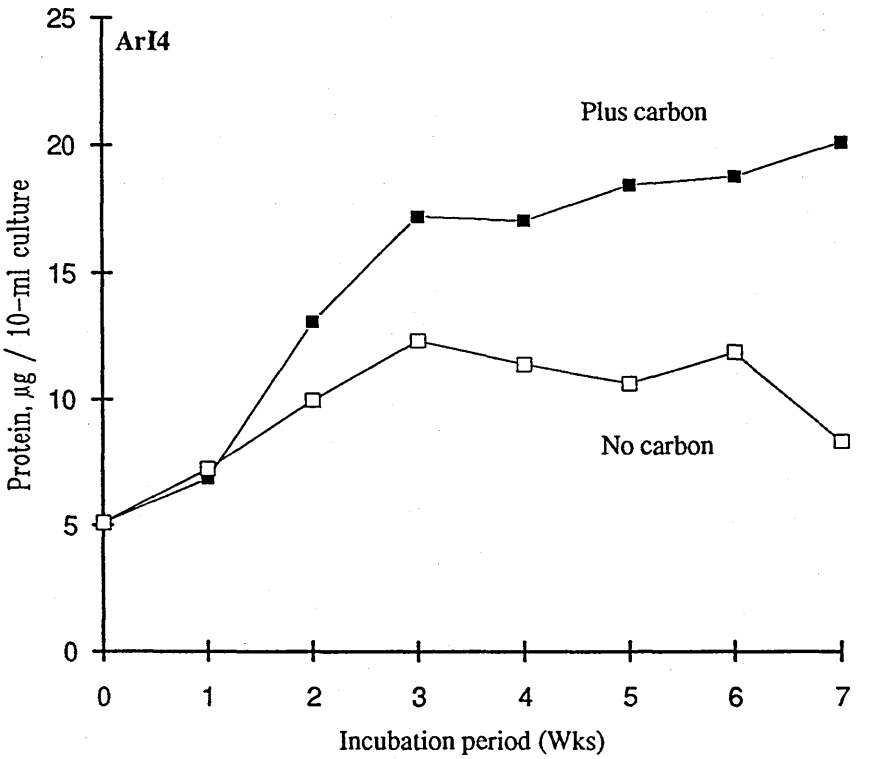


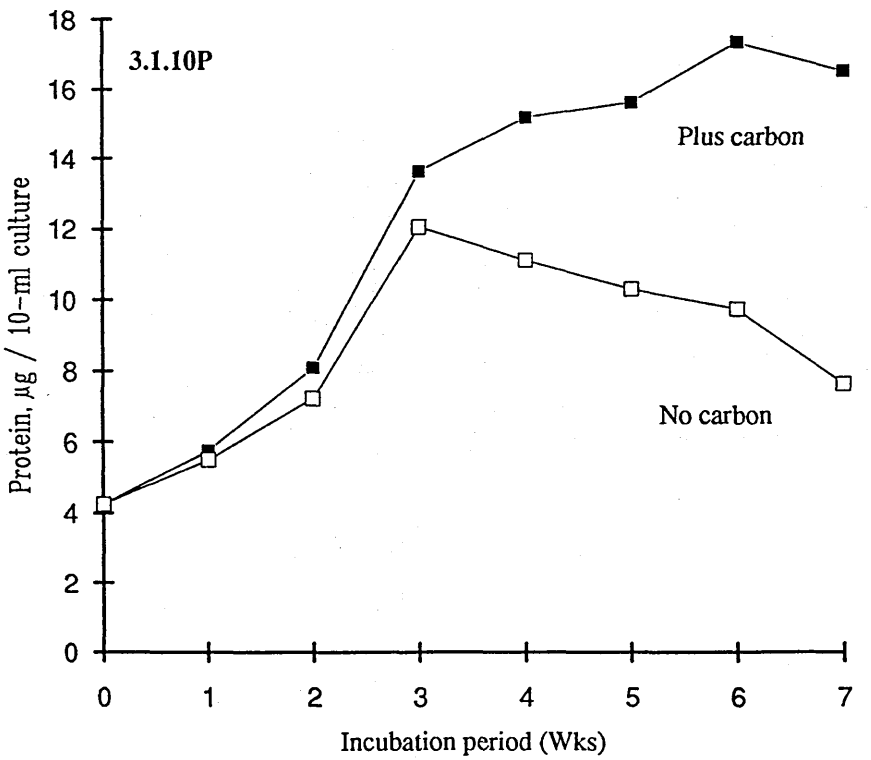
These two strains were used again in an experiment in which growth rates of *Frankia* strains growing in the presence and absence of methanol were determined (Figure 14). Both graphs show that *Frankia* grows very slowly on this C_1 compound. The growth rate of both strains was about 15% of that on propionate medium (compare with figure 7, pages 95-96).

Figure 14. Growth of two *Frankia* strains (ArI4 and 3.1.10P) in the presence and absence of methanol (5 ml/ l) as carbon source.

Frankia was dispersed as described in 3.2.4 and used to inoculate glass-tubes (2 cm x 15 cm) each containing 10 ml of the desired medium (see Materials and Methods for composition). After inoculation, tubes were covered with aluminium caps which were sealed to the tubes with "Nescofilm" and incubated in the dark under non-shaking conditions at 27 °C.

Growth was assessed weekly over a period of 7 weeks by harvesting the contents of 5 tubes of each set and determining total protein content as described in 3.2.4.


ArI4
Plus carbon
No carbon
Protein, µg / 10-ml culture
Incubation period (Wks)
0
5
10
15
20
25
0
1
2
3
4
5
6
7


3.1.10P
Plus carbon
No carbon
Protein, µg / 10-ml culture
Incubation period (Wks)
0
2
4
6
8
10
12
14
16
18
0
1
2
3
4
5
6
7

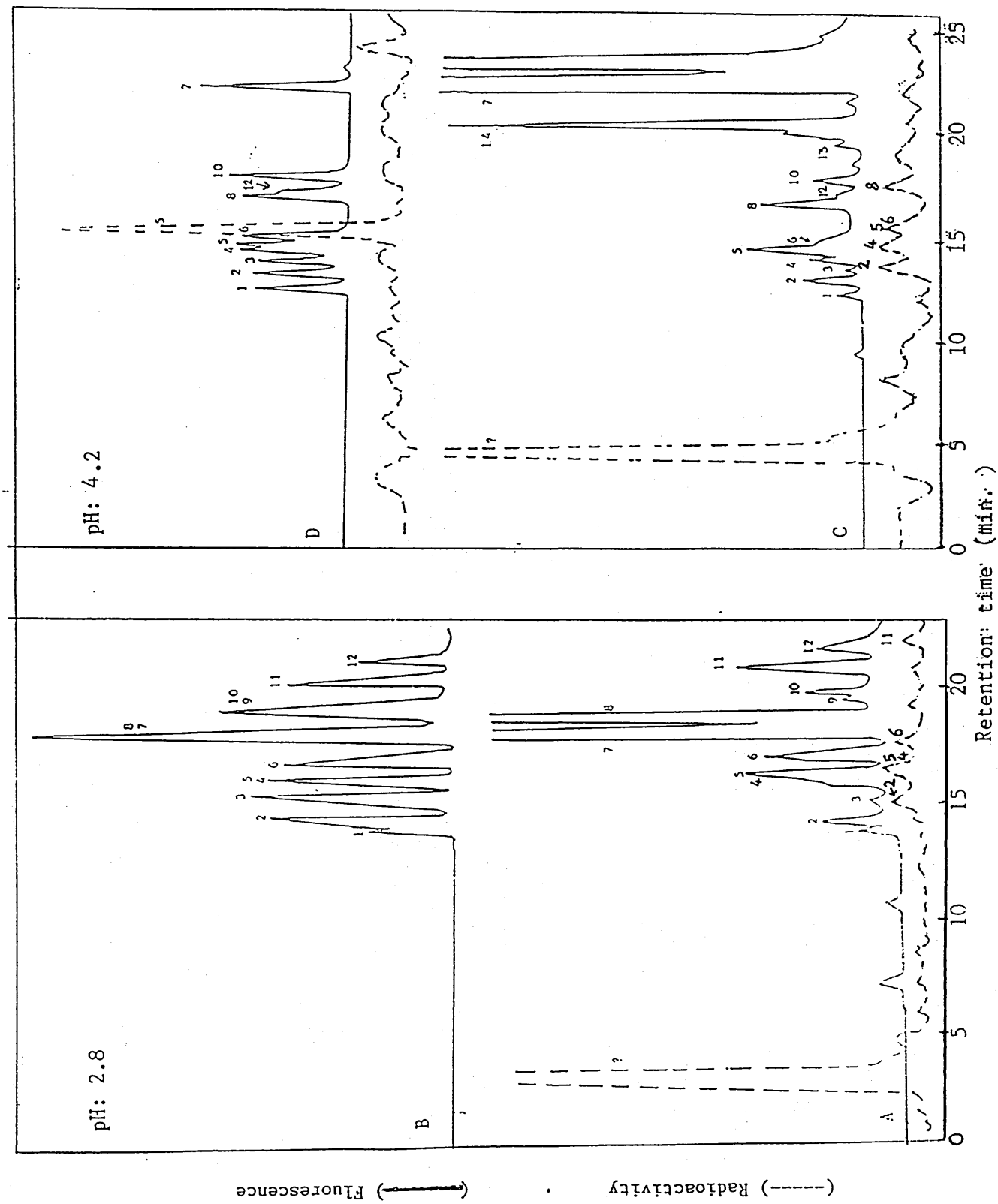
4.3.2. Metabolism of C_1 compounds.

4.3.2.1. Incorporation of ^{14}C-methanol into amino acids by methanol-grown *Frankia*.

Two mobile phase solvents (pH 2.8 and pH 4.2) for reversed-phase, gradient HPLC of amino acid and amide-FMOC derivatives, were used for analysing derivatised extracts of methanol-grown *Frankia* cultures. The main aim of this analysis was to see how much radioactivity was incorprated into serine. The use of the pH 2.8 solvent did not resolve serine and aspartic acid (Fig. 15, trace A). This separation was achieved at pH 4.2, (Fig. 15, trace C), but at the expence of the co-chromatography of serine and glutamic acid. A mixture of standard amino acids, with citrulline the only labelled amino acid, was also run in HPLC, using the pH 4.2 solvent (Fig. 15, trace D). This trace shows that the radioactive peak runs about 1 minute behind the fluorescent peak (peaks 5). Considering this result, it can be seen from trace C that: (1) very little radioactivity from ^{14}C-methanol was incorporated into serine and glutamic acid, (2) some activity was incorporated into glutamine, aspartic acid and glycine, and (3) most of the activity appeared to be in other compounds which did not co-chromatograph with amino acids.

Figure 15. Reversed-phase HPLC of FMOC derivatives of amino acids and amides using a a 0.5 M acetic acid solvent adjusted to pH 2.8 (Traces A & B) or 0.1 M acetic acid solvent adjusted to pH 4.2 (Traces C & D). Solvent programme = 0-20 min, 50-70% methanol; 20-30 min, 70-90% methanol; 30-31 min, 90-100% methanol. Traces A and C are extracts of *Frankia* (each sample contained 267.12 $dpm.\mu l^{-1}$), prepared from methanol-grown cultures as described in the Materials and Methods section. Trace B (Tonin & Wheeler, unpublished) and Trace D are mixtures of standard amino acids. The fluorescent response shown is for 1.1 nmol of each amino acid. Citrulline (peak 5) was the only radiolabelled compound in mixture D. No compound was radiolabelled in mixture B.

Labelled peaks: (1) asparagine, (2) glutamine, (3) Citrulline (4) aspartic acid, (5) serine, (6) glutamic acid, (7) FMOC-OH, (8) glycine, (9) ammonia (10) threonine, (11) γ-amino-butyric acid, (12) argenine, (13) tyrosine and (14) alanine.


pH: 4.2
pH: 2.8
A
B
C
D
Retention time (min.)
0
5
10
15
20
25
(-----) Radioactivity
(——) Fluorescence

4.3.2.2. Incorporation of ^{14}C-formaldehyde into sugar phosphates by methanol-grown *Frankia.*

Incorporation of radioactivity from ^{14}C-formaldehyde into barium acetate precipitable fractions prepared from two methanol-grown *Frankia* strains is shown in Table 14.

Table 14. Incorporation of ^{14}C-formaldehyde by cell-free extracts of methanol-grown *Frankia.*

Reaction mixture	Radioactivity incorporated (dpm.min^{-1}. mg^{-1} protein).	
	ArI4	3.1.10P
Extract + xylulose 5-phosphate	not tested	21829.8
Extract + ribose 5-phosphate	21631.5	24063.7
Sugar omitted	17398.6	15400.0

Data (means of duplicate assays) were corrected for controls where extracts were omitted from the reaction mixture.

The data show that the rate of incorporation was increased by addition of sugars to the incubation mixtures. Addition of xylulose 5-phosphate to extracts of 3.1.10P resulted in incorporation that was 10% less than that resulting from addition of ribose 5-phosphate.

The incorporation of radioactivity from ^{14}C-formaldehyde into cell-free extracts of methanol-grown *Frankia* was also examined, utilizing chromatographic techniques described in Materials and Methods. Data obtained from this experiment are illustrated in figures 16 and 17 for extracts of 3.1.10P incubated with ribose 5-phosphate or with xylulose 5-phosphate, respectively and in figure 18 for extracts of ArI4 incubated with ribose 5-phosphate.

It can be seen that the complete mixtures (i.e., chromatogram B of each figure), with either strain, fixed much more radioactivity into the area which has similar R_f as the phosphate sugars, than did the controls (chromatograms labelled A). Another striking feature of the pattern of radioactivity distribution is that in each

figure the complete mixture contained much more radioactivity in the origin area than did reactions without added sugars.

When the complete mixtures were treated with an acid phosphatase (chromatograms labelled C) much of the radioactivity appeared in a region with R_f similar to that of free sugars standards. Furthermore, the origin areas of the phosphatase-treated mixtures contained very little radioactivity, compared to that of the complete mixtures. However, for unknown reasons, the total radioactivity that appeared in chromatogram C in the case of ArI4 strain was very high compared with that of chromatogram B.

Figure 16. The distribution of radioactivity on thin-layer chromatograms (TLC) of extracts of methanol-grown 3.1.10P *Frankia* strain following incubation with ^{14}C-formaldehyde and ribose 5-phosphate. A: represents the control (i.e., ribose 5-P was omitted from the reaction mixture). B: represents the complete reaction mixture (see Materials and Methods for details of composition). C also represents the complete reaction mixture but chromatographed after treatment with acid phophatase. Along the top of B are shown the chromatographic locations of phosphorylated sugars and along the top of C are shown the chromatographic locations of free sugars that resulted from treatment of phosphorylated sugars with acid phosphatase.

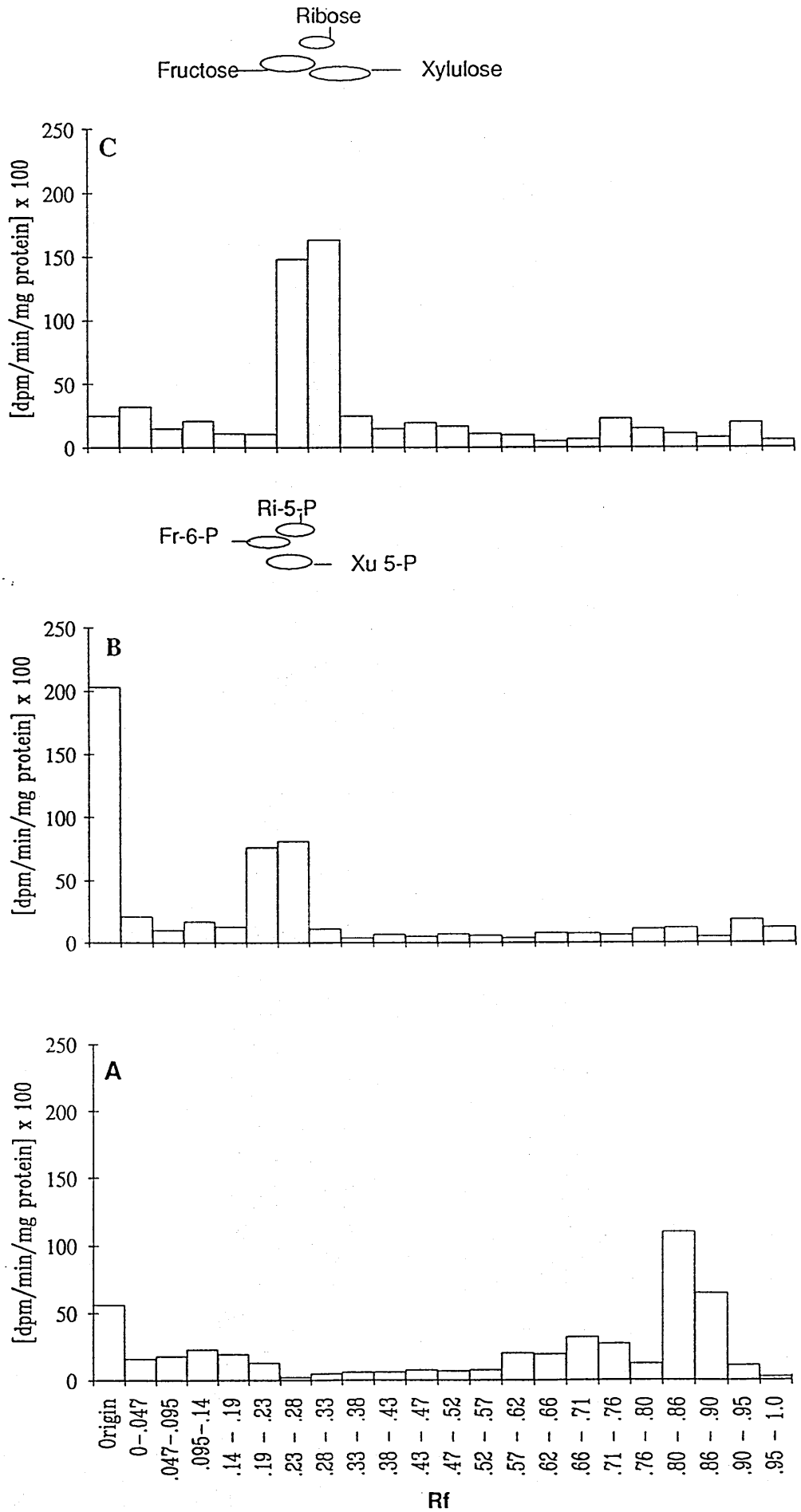

Ribose
Fructose
Xylulose
C
[dpm/min/mg protein] x 100
250
200
150
100
50
0
Ri-5-P
Fr-6-P
Xu 5-P
B
A
Origin
0-.047
.047-.095
.095-.14
.14 - .19
.19 - .23
.23 - .28
.28 - .33
.33 - .38
.38 - .43
.43 - .47
.47 - .52
.52 - .57
.57 - .62
.62 - .66
.66 - .71
.71 - .76
.76 - .80
.80 - .86
.86 - .90
.90 - .95
.95 - 1.0
Rf

Figure 17. The distribution of radioactivity on thin-layer chromatograms (TLC) of extracts of methanol-grown 3.1.10P *Frankia* strain following incubation with ^{14}C-formaldehyde and xylulose 5-phosphate. A: represents the control (i.e., xylulose 5-P was omitted from the reaction mixture). B: represents the complete reaction mixture (see Materials and Methods for details of composition). C also represents the complete reaction mixture but chromatographed after treatment with acid phophatase. Along the top of B are shown the chromatographic locations of phosphorylated sugars and along the top of C are shown the chromatographic locations of free sugars that resulted from treatment of phosphorylated sugars with acid phosphatase.

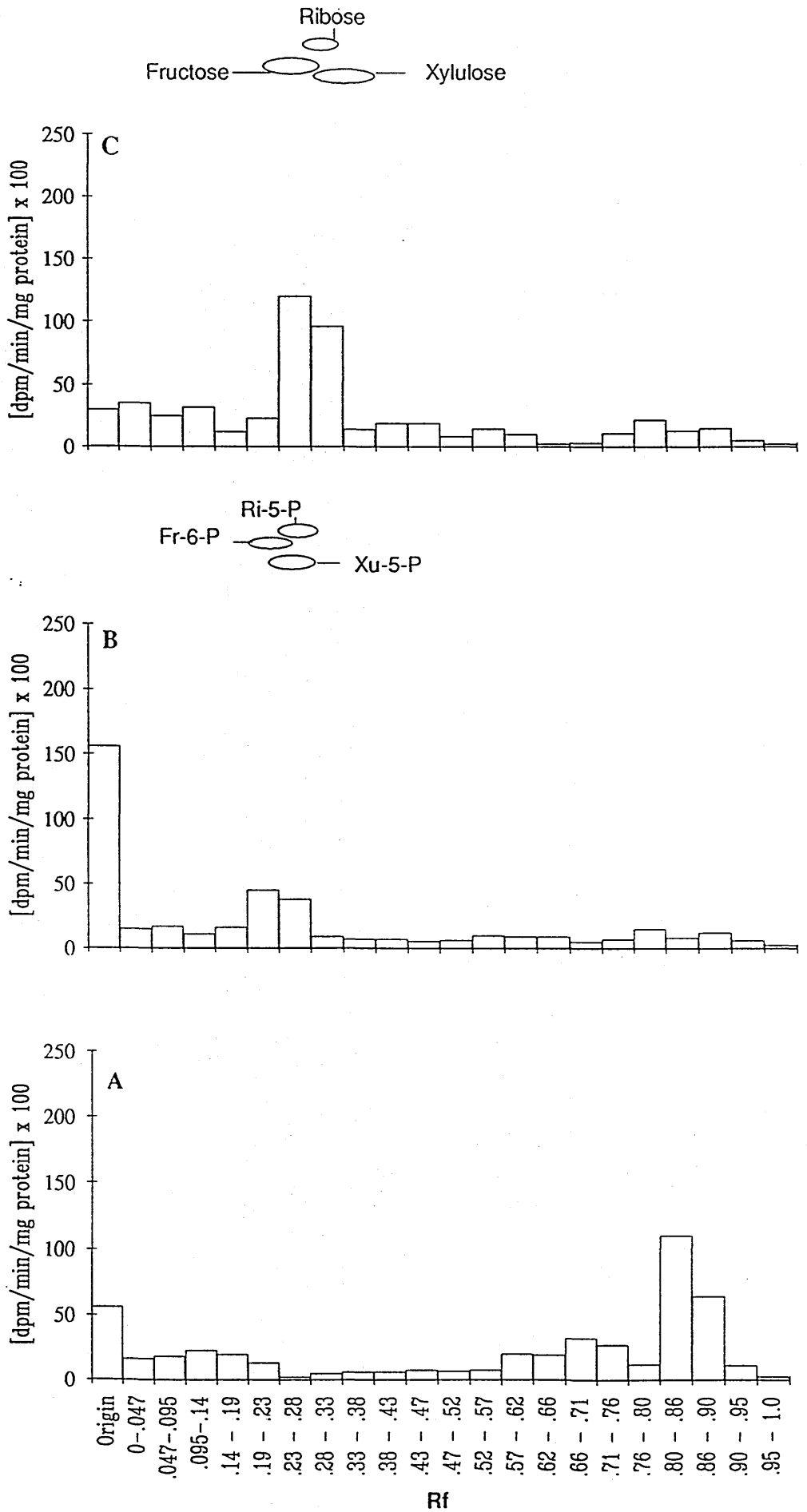
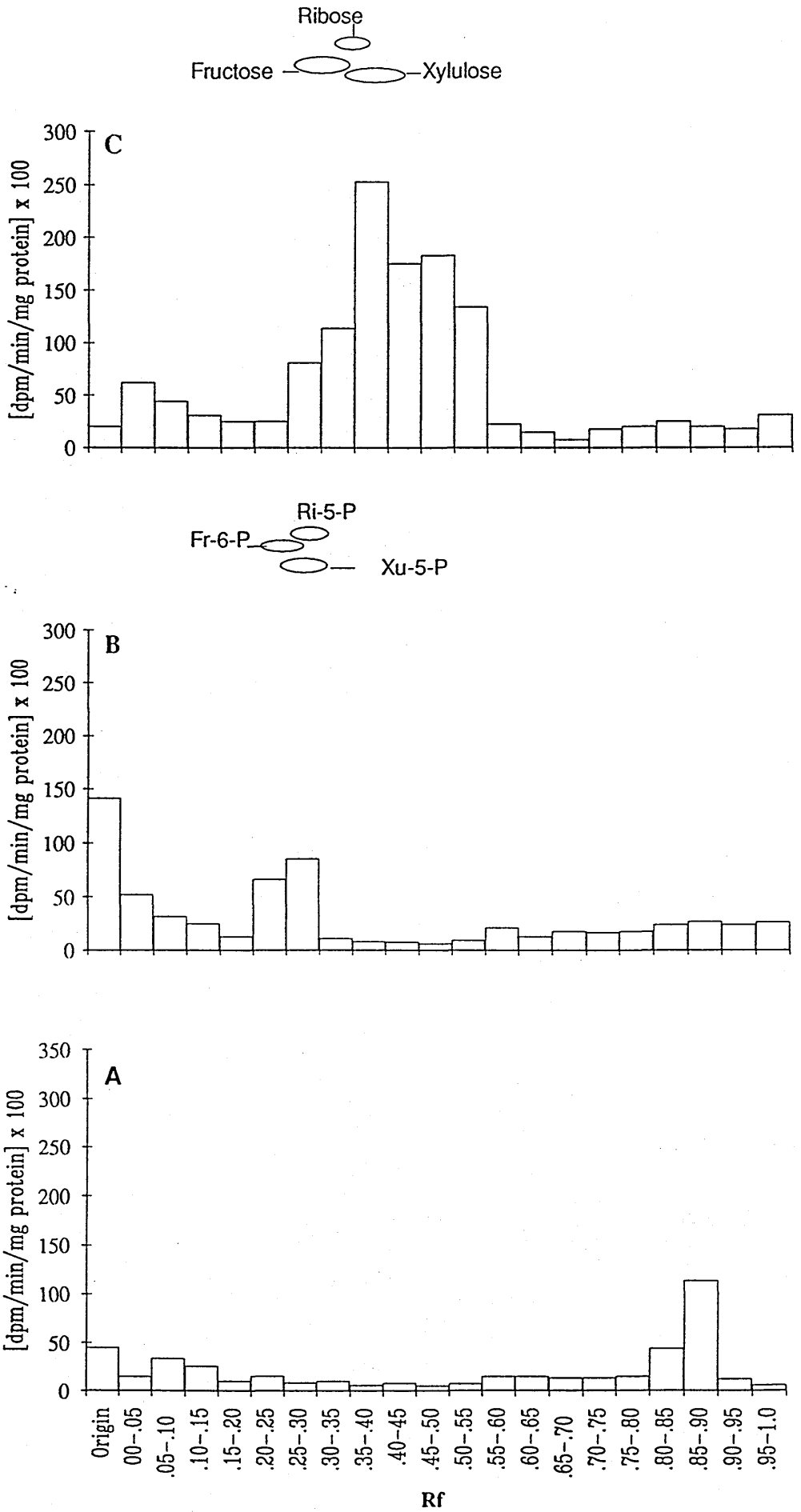

Ribose
Fructose
Xylulose
C
[dpm/min/mg protein] x 100
250
200
150
100
50
0
Ri-5-P
Fr-6-P
Xu-5-P
B
A
Origin
0-.047
.047-.095
.095-.14
.14 - .19
.19 - .23
.23 - .28
.28 - .33
.33 - .38
.38 - .43
.43 - .47
.47 - .52
.52 - .57
.57 - .62
.62 - .66
.66 - .71
.71 - .76
.76 - .80
.80 - .86
.86 - .90
.90 - .95
.95 - 1.0
Rf

Figure 18. The distribution of radioactivity on thin-layer chromatograms (TLC) of extracts of methanol-grown ArI4 *Frankia* strain following incubation with ^{14}C-formaldehyde and ribose 5-phosphate. A: represents the control (i.e., ribose 5-P was omitted from the reaction mixture). B: represents the complete reaction mixture (see Materials and Methods for details of composition). C also represents the complete reaction mixture but chromatographed after treatment with acid phophatase. Along the top of B are shown the chromatographic locations of phosphorylated sugars and along the top of C are shown the chromatographic locations of free sugars that resulted from treatment of phosphorylated sugars with acid phosphatase.


Ribose
Fructose
Xylulose
C
[dpm/min/mg protein] x 100
300
250
200
150
100
50
0
Ri-5-P
Fr-6-P
Xu-5-P
B
[dpm/min/mg protein] x 100
300
250
200
150
100
50
0
A
[dpm/min/mg protein] x 100
350
300
250
200
150
100
50
0
Origin
00–.05
.05–.10
.10–.15
.15–.20
.20–.25
.25–.30
.30–.35
.35–.40
.40–.45
.45–.50
.50–.55
.55–.60
.60–.65
.65–.70
.70–.75
.75–.80
.80–.85
.85–.90
.90–.95
.95–1.0
Rf

4.4. DISCUSSION.

Contrary to the long-held view that *Frankia* does not exhibit extra-nodular growth, it is now almost certain that like the majority of actinomycetes *Frankia* also is able to grow saprophytically in soil (see Introduction). It is also well known that C_1 compounds such as methane, and methanol which arises from methane, are widely distributed in habitats common for some actinorhizal species. Thus, there is a strong possibility that like some other actinomycetes *Frankia* may be able to utilize these C_1 compounds as sole carbon source for growth.

Preliminary data supporting this possibility have been obtained here. The stimulation of growth (based on total protein yield) of 6 from 8 and 3 from 3 strains tested when methanol and formate, respectively, were supplied singly as sole carbon source in the growth medium, indicates the ability of strains to grow on these C_1 compounds (Table 13, Fig. 12, and Fig. 14).

As reviewed in the introduction, there are three pathways by which methylotrophic bacteria synthesizes polycarbon compounds from C_1 units. The most common of these is the so-called Calvin cycle. In this pathway, C_1 growth substrates are oxidised to CO_2 which is then fixed *via* the action of the ribulose bis-phosphate carboxylase (RuBPc) enzyme (see page 118). However, results obtained in Chapter 3 of this thesis suggest strongly that this pathway does not operate in *Frankia*. This conclusion is drawn from: (a) the failure of all *Frankia* strains tested to grow autotrophically with 5% CO_2 + 5% H_2 (Table 10) and (b) the failure to detect activity for the key enzyme (RuBPc) of this pathway in cell-free extracts of *Frankia* (Table 11).

Another pathway used by methylotrophic bacteria when growing on C_1 substrates is the serine pathway. In this pathway, previous studies (Kaneda & Roxburgh, 1959_a; Large *et al.*, 1961) have shown that the first stable products of [^{14}C]methanol fixation were serine (about 50% of total carbon fixed) and C_4 dicarboxylic acids such as aspartate and malate. Other radiolabelled products were

glycine and some phosphorylated compounds. The HPLC analysis of extracts of some cultures fed with ^{14}C-methanol (Fig. 15) showed that little radioactivity from ^{14}C-methanol was incorporated into serine. This apparent absence of high levels of incorporation of radioactivity into serine was suggestive of metabolism of methanol by a different route in *Frankia*. The detection of some activity in aspartic acid, glycine and other unidentified compounds which did not co-chromatograph with amino acids is in agreement with the results of Johnson & Quayle (1965) for methane-grown *Pseudomonas methanica* (in the event, the ribulose mono-phosphate pathway was found to operate in this organism).

Since its first discovery by Johnson & Quayle (1965), the ribulose mono-phosphate pathway has been shown to operate in a wide range of methylotrophic bacteria (see introduction). In view of the failure to obtain positive indications for operation of the serine pathway in methanol-grown *Frankia* strains and the early elimination of a possible involvement of the Calvin cycle, the possible operation of the ribulose mono-phosphate pathway during assimilation of methanol by these strains was examined.

The ideal way to provide conclusive evidence for the operation of this pathway in any methylotrophic bacterium would be to carry out assays for activities of both the hexulose phosphate synthase and hexulose phosphate isomerase, key enzymes of this pathway (Large, 1983; Large & Bamforth, 1988), in cell-free extracts of C_1-grown cultures. However, at the time of this experiment, I was unable to obtain phosphohexuloisomerase and D-arabino 3-hexulose 6-phosphate, to carry out the assays according to published method of Van Dijken *et al.* (1978). Alternatively, earlier methods used by Kemp & Quayle (1966) and Lawrence *et al* (1970) were used as described in the Materials and Methods section.

The presence of hexose phosphate synthase in extracts of methanol-grown ArI4 and 3.1.10P *Frankia* strains was suggested by the stimulation by the addition of ribose 5-phosphate of incorporation of substantial radioactivity from H^{14}CHO into a barium acetate precipitable fraction, containing sugar phosphates (Table 14). This

result was interpreted as indicating that *Frankia* assimilates methanol *via* the hexulose mono-phosphate pathway, an interpretation reinforced by the detection in *Frankia* of 6-phosphogluconate dehydrogenase (Vikman & Huss- Danell, 1987_a), a key enzyme of this pathway (Large & Bamforth, 1988).

Further support for this interpretation was obtained from the change in the mobility on thin layer chromatograms (TLC) of part of this radioactivity following treatment of the incubation mixture with acid phosphatase (Figures, 16 to 18).

The pattern of distribution of radioactivity in chromatograms B of figures 16 and 18, which represent the complete reaction mixtures, suggest that condensation of formaldehyde with ribose 5-phosphate catalysed by extracts of either strain leads to the formation of two phosphorylated compounds which have the chromatographic properties of phosphorylated sugars. Treatment of these mixtures with acid phosphatase and co-chromatography with free sugar standards (chromatograms C) suggest that one of these compounds is fructose. Similar results have been reported by Quayle and co-workers in *Pseudomonas* and *Methanomonas* species when using the HMP pathway for assimilation of C_1 growth substrates (these are reviewed in the Introduction). According to these researchers the second compound is hexulose. Treatment of the mixtures with acid phosphatase lead also to the movement of most of the radioactivity that remained in the origin area of untreated mixtures. This could be attributed to: (a) some of the reaction products were insoluble in methanol (diluent of the complete mixtures) but were soluble in water (diluent of treated mixtures), or (b) the action of phosphatase itself since Kemp & Quayle (1966) and Lawrence *et al.* (1970) noted that after treatment of the complete mixture with acid phosphatase in the first place, considerable radioactivity remained near the origin, but when these origin areas were eluted, treated with phosphatase and re-chromatographed more radioactivity appeared in the free sugar area. From this, they concluded that a large proportion of the radioactivity remaining in the origin area arose from the incompleteness of dephosphorylation by the acid phosphatase preparation after the first treatment. These researchers did not report the

concentration of acid phosphatase used in their experiments (cf. 5 $mg.ml^{-1}$ used in the present study). Nevertheless, neither of these two interpretations explain the appearance of more radioactivity all over the chromatogram when the complete mixture containing ArI4 extract was treated with phosphatase, compared to the untreated mixture (Fig. 18). This could be due to an experimental error in the application of aliquots of extracts to the chromatogram. This experiment was done at the end of my research period so time was not available to verify this.

Replacement of ribose 5-phosphate with xylulose 5-phosphate in the reaction mixtures containing extracts of 3.1.10P strain (Fig. 17) resulted in much less radioactivity incorporation, particularly in the phosphorylated area, than that resulting from the addition of ribose 5-phosphate. Nevertheless, the pattern of radioactivity distribution on the chromatogram was very similar to that obtained with ribose 5-phosphate. This could be interpreted as suggesting that some of the xylulose 5-phosphate added to the mixture was epimerased to ribose 5-phosphate *via* pentose phosphate epimerase. The resulting ribose 5-phosphate was then used in the reaction. The possibility of condensation of formaldehyde with xylulose 5-phosphate here *via* the Xylulose mono-phosphate pathway (XuMP) of formaldehyde fixation, which usually operates in yeasts, is ruled out. This is because the second step which involves the formation of fructose 1,6 bis-phosphate, the precursor of fructose 6-phosphate, is ATP dependent (Large & Bamforth, 1988).

It is of interest that all 3 strains tested will grow with formate as C source. Oxidation of formate to CO_2 will provide energy and reductant for growth. At the same time, CO_2 released by this reaction must be utilized by other carboxylation reactions (eg., catalysed by PEPc) to re-synthesise organic carbon for growth. Furthermore, as already mentioned in the introduction, a number of investigations have demonstrated that under certain growth conditions some organisms may change the assimilatory pathway used for C_1 assimilation (some examples have been given in page 116).

The present study is indicative but not conclusive evidence for the involvement of the hexulose phosphate synthase in the fixation of formaldehyde in *Frankia*. Clearly, further data will be needed for the establishment of the suggested route for assimilation of methanol in *Frankia*. However, the results presented here fulfils part of the requirement for an operational HMP pathway and may now open up a new direction in the study of carbon nutrition in *Frankia*.

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