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Physiological Aspects of Microbial Inorganic Nitrogen Metabolism

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I. Introduction

In this article we have set out to provide a comparative review of the known mechanisms of inorganic nitrogen assimilation in free-living micro-organisms. We have excluded from consideration the assimilation of all other (organic) nitrogen sources but, since the breakdown of these compounds (e.g. amino acids, amides, urea) generally yields ammonia, these also may be assimilated by some of the mechanisms described below. To the best of our knowledge, the major pathways of inorganic nitrogen assimilation are those shown in Fig. 1, with ammonia occupying a central position as intermediate in the assimilation of both molecular nitrogen and nitrate. The direct assimilation of nitrate to form first nitropropionic acid is known to occur in some fungi (see Painter, 1970) but it is doubtful whether this, or analogous systems, are of widespread significance in micro-organisms.

Ammonium ions dissociate to form ammonia and hydrogen ions at alkaline pH values, the pK of this reaction being about 9·2. As the true substrate for many of the reactions described in this article is unknown, we have used the term "ammonia" throughout to denote the assimilated substrate, be it NH_3 or NH_4^+.

II. Assimilation of Molecular Nitrogen

Nitrogen fixation by free living organisms has been the subject of a number of reviews in recent years (Hardy and Burns, 1968; Chatt and Fogg, 1969; Postgate, 1970, 1971; Benemann and Valentine, 1972; Dalton and Mortenson, 1972). This section will therefore deal only with the overall physiology of the process.

Early workers used the measurement of nitrogen gain (determined by the Kjeldahl method) after growth in a medium free of fixed nitrogen as evidence for nitrogen fixation, but this was superseded by techniques employing enrichment with the stable isotope ^{15}N (Burris *et al.*, 1943). The ^{15}N method proved to be about 100-times more sensitive than the Kjeldahl procedure and, using this technique, the list of nitrogen-fixing organisms steadily increased. Efforts were also concentrated on the characterization of the "key intermediate" in nitrogen fixation (Wilson and Burris, 1953), defined as the inorganic product of the fixation reaction via which fixed nitrogen was assimilated into a carbon skeleton. Ammonia soon became the most likely candidate and the evidence for the involvement of this compound has been reviewed by Wilson and Burris (1953), Nicholas (1963a, b) and Wilson (1969). Such evidence includes the observation that ammonia may be used without lag by organisms fixing nitrogen. Furthermore, ammonia has been isolated as a product of ^{15}N fixation in cultures of *Clostridium pasteurianum* (Zelitch *et al.*, 1951) and *Azotobacter vinelandii* (Newton *et al.*, 1953). It is of interest to note that in *Cl. pasteurianum*, while about 50% of the ^{15}N fixed accumulated in the culture medium as ammonia (which always contained the highest labelling activity), the compound with the second highest activity was glutamine (amide nitrogen); this is in agreement with recent evidence concerning the mode of ammonia assimilation in this organism (Dainty and Peel, 1970; Dainty, 1972). Finally, cell-free extracts of a number of organisms showed conversion of ^{15}N to $^{15}NH_3$ and, indeed, nitrogen fixation has been assayed as ammonia production in several instances (e.g. Mortenson, 1962; Munson and Burris, 1969). In extracts of *Cl. pasteurianum* (Carnahan *et al.*, 1960a, b) incorporated ^{15}N was recovered quantitatively as ammonia and the normal yield of the product was of the order 30 μg ammonia nitrogen/ml. The possible pathways involved in the reduction of nitrogen to ammonia have been reviewed

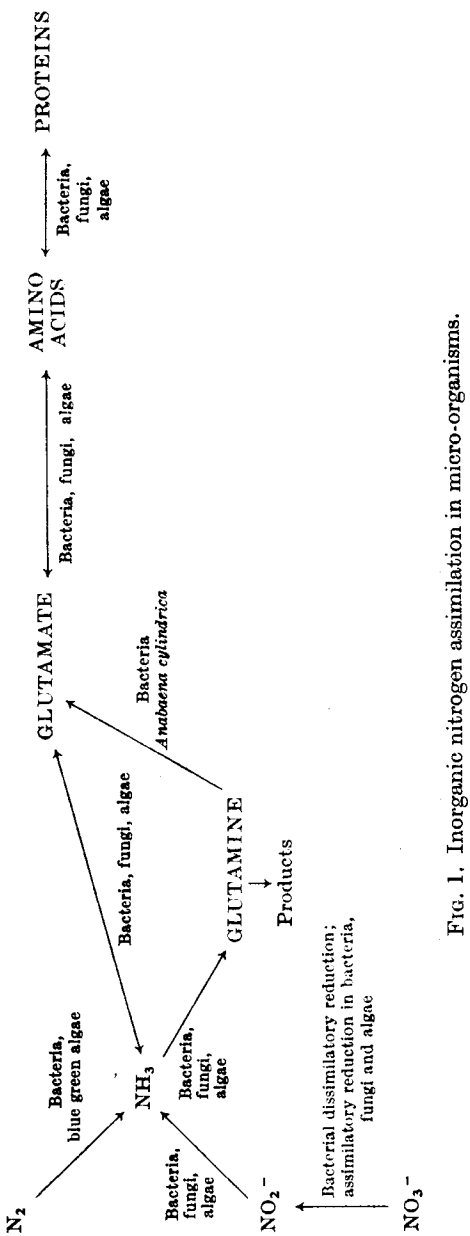

FIG. 1. Inorganic nitrogen assimilation in micro-organisms.

recently by Benemann and Valentine (1972) and will not be further elaborated here.

A useful property of the nitrogen-fixing enzyme complex (nitrogenase) is that substrates other than nitrogen also may be reduced. Such substrates include nitrous oxide, the azide and cyanide ions, methyl isocyanide and acetylene (see Hardy and Burns, 1968; Burris, 1969). The ATP-dependent reduction of any of these substrates is specifically associated with the ability to fix nitrogen and this property is absent from cultures in which synthesis of the enzyme system involved has been repressed by growth on fixed nitrogen. The reduction of acetylene (Schöllhorn and Burris, 1966; Dilworth, 1966) is of particular importance since the product (ethylene) may be detected in very small quantities using gas chromatography (Postgate, 1972) and the "acetylene reduction test" is now widely used for screening possible nitrogen-fixing organisms, for the assessment of nitrogen fixation in natural environments and for the assay of nitrogenase (the enzyme system involved in nitrogen fixation) in whole cells and cell extracts.

By the combined methods of ^{15}N incorporation and the acetylene reduction test, the present day list of *bona fide* free-living nitrogen fixing organisms is small, and restricted to a relatively few species of prokaryotic organisms (Stewart, 1969; Postgate, 1971). Reports of nitrogen-fixing yeasts and higher fungi have been discounted by the painstaking work of Millbank (1969, 1970). Nitrogen-fixing bacteria include obligate aerobes of the family Azotobacteriaceae and *Mycobacterium flavum*, facultative anaerobes such as *Klebsiella pneumoniae* and *Bacillus polymyxa* (which fix nitrogen only under anaerobic conditions) and obligate anaerobes such as *Cl. pasteurianum*, *Desulfovibrio desulfuricans* and *Desulfomaculum ruminis*. There are also authenticated reports of nitrogen fixation in photosynthetic bacteria such as *Rhodospirillum rubrum*, *Chromatium* and *Chloropseudomonas ethylicum*, while positive acetylene reduction has been reported in the acidophilic *Thiobacillus ferrooxidans* (Mackintosh, 1971). Dixon and Postgate (1971) succeeded in transferring the genes responsible for nitrogen fixation (nif) by conjugation between mutant strains of *K. pneumoniae*, and followed this with a similar transfer between *K. pneumoniae* and *Escherichia coli* (Dixon and Postgate, 1972). This development opens up the possibility of producing a large number of derived nitrogen-fixing organisms, and this could be of tremendous ecological and, possibly, economic significance. In a survey of blue-green algae, Stewart (1969) lists some 40 species known to fix nitrogen, all being filamentous and heterocystous members of the families Nostocaceae, Scytonemataceae, Stigonemataceae and Rivulariaceae. To this list must now be added the filamentous, non-heterocystous *Plectonema boryanum* (Stewart and Lex

1970) and two strains of the unicellular *Gleocapsa* (Wyatt and Silvey, 1969; Rippka *et al.*, 1971).

A. Bacterial Nitrogen Fixation

Nitrogen fixation is essentially an anaerobic process and, when resolved into its component proteins, the particulate nitrogenase of *Azotobacter* was found to be as sensitive to oxygen as the soluble enzyme preparations from anaerobes. *Azotobacter* must therefore possess mechanisms for allowing the oxygen-sensitive reactions of nitrogen fixation to occur during aerobic growth. However, although *Azotobacter* sp. are obligate aerobes, nitrogen-fixing cultures are inhibited by excessive aeration; and this sensitivity is not shown by cultures grown on ammonia (Dalton and Postgate, 1969a, b). In a similar way *Mycobacterium flavum* grows best at low oxygen tensions when fixing nitrogen (Biggins and Postgate, 1969), as does *Derxia gummosa* (Hill and Postgate, 1969).

As discussed by Postgate (1971) and Hill *et al.* (1972) two regulatory processes are thought to occur in *Azotobacter* sp. The first is the process of "respiratory protection" in which oxygen is probably excluded from the site of nitrogen fixation by the high rate of respiration characteristic of these organisms. The second process, which has been termed "conformational protection", occurs when, for some physiological reason (e.g. high aeration or carbon limitation), respiratory protection is inadequate. Under these conditions, a conformational change probably takes place in the enzyme complex so that the oxygen-sensitive sites become inaccessible to oxygen (but concomitantly lose their enzymic activity). Thus the particulate nitrogenase preparations from *A. vinelandii* may exist in an oxygen-insensitive form (Bulen *et al.*, 1965) unlike the soluble preparations from the anaerobe *Cl. pasteurianum* which are always oxygen sensitive. As the particulate nitrogenase enzyme system of *A. vinelandii* was oxygen sensitive when resolved into its component parts (Bulen and Le Compte, 1966), Dalton and Postgate (1969a, b) postulated that the oxygen-tolerant nitrogenase particle represented a model of the "conformationally protected" enzyme. Oppenheim and Marcus (1970a, b) have shown that *A. vinelandii* grown on molecular nitrogen possess an extensive internal network of membranes which is absent from cells grown on ammonia. The assumption is that this membrane system protects the particulate nitrogenase from damage by oxygen and, therefore, provides a site for the process of conformational protection. Drozd *et al.* (1972) have grown *A. chroococcum* in chemostat cultures in which nitrogenase synthesis was fully derepressed, partly repressed or fully repressed depending on the concentration of ammonia

added to the medium. As in *A. vinelandii*, the convoluted internal membrane system present in nitrogen-fixing cells was absent from those repressed by ammonia although, perhaps surprisingly, the phospholipid content of both types of cell was similar. All members of partly repressed cultures possessed some internal membranes but in smaller amounts than in fully derepressed cells.

The first bacterial cell-free nitrogen-fixing system (Carnahan *et al.*, 1960a, b) was obtained with extracts of *Cl. pasteurianum* prepared either by Hughes press treatment or autolysis of dried cells. The soluble (not sedimented at 144,000 g after 2 hours) oxygen-sensitive system utilized pyruvate which was metabolized by phosphoroclastic cleavage. This process provided the enzyme system with the two prerequisites for nitrogen fixation, a source of reducing power and a source of ATP. Bulen *et al.* (1965) showed that sodium dithionite could act as electron donor in cell-free preparations from *A. vinelandii* and *Rhodospirillum rubrum*. Since this report, dithionite has been widely used in cell-free systems together with either ATP or an ATP-generating system (creatine phosphate and creatine kinase). The employment of dithionite, while being useful in determining many characteristics of nitrogenase, gave no information on the nature of the physiological electron donors. Mortenson *et al.* (1962), however, found that in extracts of *Cl. pasteurianum* an electron carrier of low potential was involved linking pyruvate utilization to nitrogen fixation. This carrier protein, which contained non-haem iron, was termed ferredoxin. Crude extracts of *K. pneumoniae* and *B. polymyxa* will also fix nitrogen in the presence of pyruvate while those of *A. vinelandii* will not, although all four organisms will utilize reduced nicotinamide nucleotides as electron donors. Ferredoxins have been implicated as electron carriers during nitrogen fixation in *B. polymyxa* and *A. vinelandii* as well as in *Cl. pasteurianum* (see Benemann and Valentine, 1972).

As pointed out by Postgate (1971), reduction of nitrogen to ammonia could in theory be exergonic, and yet nitrogen fixation has a strict requirement for ATP (the ATP/2e ratios obtained with purified extracts are 4·3 for *Azotobacter* and 3·0 for *Clostridum*; Dalton and Mortensen, 1972). The role of ATP is obscure although, with purified components of *Cl. pasteurianum* and *K. pneumoniae*, ATP binding to nitrogenase proteins could be demonstrated (Bui and Mortenson, 1968; Biggins and Kelly, 1970) and it was therefore assumed that this brought the ATP into a suitable complex for its utilization in the reduction process (perhaps for electron activation). This requirement for ATP may be demonstrated by the lower molar growth yields obtained in organisms fixing nitrogen relative to those utilizing ammonia. Hill *et al.* (1972) have compared the yields of *A. chroococcum*, *Kl. pneumoniae*, *Cl. pasteurianum*

and *Desulfovibrio desulfuricans* in carbon and energy-limited chemostat cultures and, in every instance, the yield obtained when grown on molecular nitrogen was much less than when grown on ammonia.

Nitrogenase preparations from a number of bacteria have been purified and fractionated into their component proteins. Purification usually involved anaerobic ion-exchange chromatography and, using this method, two distinct protein moieties were resolved, one sensitive to oxygen and the other less so. Nitrogenase proteins from *A. vinelandii*, *K. pneumoniae* and *Cl. pasteurianum* were similar in their general properties and in the conditions they required for activity (for discussion see Postgate, 1971, Eady *et al.*, 1972). Protein 1 was the oxygen insensitive component which contained both molybdenum and non-haem iron in an average ratio of approximately 1 : 17. Protein 1 from all three organisms was made up of sub-units and, while the aggregate protein differed with respect to its molecular weight in different bacteria, there was a close similarity in specific activities. Protein 2 was the oxygen-sensitive component and, while containing non-haem iron, did not contain molybdenum. Protein 2 of *K. pneumoniae* and *Cl. pasteurianum* varied in size but both were made up of sub-units and contained iron and acid-labile sulphide in equivalent amounts. In *K. pneumoniae*, reduction of both nitrogen and acetylene was maximal when proteins 1 and 2 were present in a 1 : 1 molar ratio (Eady *et al.*, 1972). Proteins 1 and 2 from a number of bacteria showed some cross reactivity (Detroy *et al.*, 1969; Kelly, 1969). Thus proteins from *A. chroococcum* would substitute for those of *K. pneumoniae* and those of *K. pneumoniae* with those of *B. polymyxa* with about 80% maximum activity. The cross reactivity shown between proteins of *A. chroococcum* and *B. polymyxa*, and *A. vinelandii* and *B. polymyxa*, were much less, however, while those of *B. polymyxa* and *Cl. pasteurianum* were inactive. These cross reactivities may reflect evolutionary relationships between the organisms involved.

Nitrogen fixation in cell-free extracts of the photosynthetic bacterium *R. rubrum* was first reported by Schneider *et al.* (1960) and later Bulen *et al.* (1965) showed that enzyme activity was stimulated on addition of pyruvate. Burns and Bulen (1966) found that, in extracts of *R. rubrum* prepared by sonication or French press treatment, nitrogenase activity was present in a 144,000 g supernatant. Recently Schick (1971) determined nitrogen fixation in whole cells of *R. rubrum* manometrically, and concluded that a range of environmental conditions (including light intensity, pH value and temperature) influenced nitrogen uptake, and further reported that pyruvate stimulated uptake (10 mol pyruvate being consumed per mol nitrogen "fixed"). Cell-free nitrogen fixation has also been obtained with extracts of the purple sulphur bacterium *Chromatium* (Winter and Arnon, 1970, Yoch and Arnon, 1970) and in the

green heterotrophic bacterium (*Chloropseudomonas ethylicum* (Evans and Smith, 1971). Winter and Arnon (1970) showed that, in extracts of *Chromatium* prepared by sonication, reduction of either nitrogen or acetylene required reducing power and ATP. Reducing power could be supplied by dithionite, reduced ferredoxin or by hydrogen in the presence of catalytic amounts of viologen dye. It was further reported (Yoch and Arnon, 1970) that the ATP requirement could be supplied by photosynthetic phosphorylation although the direct photoreduction of nitrogen was not demonstrated. Evans and Smith (1970) reported a soluble (150,000 g) nitrogenase in sonicated extracts of *Chloropseudomonas ethylicum*. In the intact organism, acetylene reduction was found to be light dependent; activity in the dark was only 10% of that in the light. In crude extracts, pyruvate produced a faster rate of reduction than did dithionite. Fractionation of the crude extract on DEAE cellulose removed ferredoxin and, in extracts so treated, pyruvate-dependent acetylene reduction occurred only in the presence of added ferredoxin. Ferredoxin photoreduced in the presence of photosynthetic particles from the same organism, or with illuminated spinach chloroplasts, served as electron donor for acetylene reduction in the presence of an ATP-generating system. Ferredoxins from *Chromatium* or *Cl. pasteurianum* were less effective than those from the parent organism.

The overall requirements for nitrogen fixation may be considered to be the presence of an adequate supply of substrates, the absence of inhibitors and a suitable environment. Substrates include molecular nitrogen, a supply of ATP provided by an active metabolism, and carbon skeletons to accept the product of nitrogenase action (ammonia). Inhibitors include ammonia, which represses synthesis of nitrogenase, and ADP which, in *Cl. pasteurianum* at least, inhibits nitrogenase activity. Environmental factors include the oxygen tension, for, even with aerobic bacteria and blue-green algae (see below), nitrogen fixation is most efficient at low dissolved oxygen tension. In a natural aquatic or soil environment, fixation by non-photosynthetic bacteria is probably limited by the availability of sources of carbon and energy, and for this reason, nitrogen fixation by photosynthetic bacteria and blue-green algae is usually considered to be of greater significance.

There is general agreement that some fixed nitrogen sources (including nitrate and ammonia) repress the synthesis of nitrogenase in bacteria and that nitrogen itself, at least in more than trace amounts, is not required for nitrogenase synthesis (Hill *et al.*, 1972; Dalton and Mortenson, 1972; Benemann and Valentine, 1972; Drozd *et al.*, 1972). Thus nitrogenase was absent from ammonia-grown cultures of *Azotobacter* (Bulen *et al.*, 1964) and did not appear in cultures of *Azotobacter* or *Cl. pasteurianum* until ammonia was exhausted (Strandberg and Wilson, 1967; Daesch and Mortenson, 1968). In both *K. pneumoniae* and *A.*

vinelandii diauxic growth occurred when cultures were grown first on ammonia and then on molecular nitrogen and it was assumed that nitrogenase synthesis occurred during the diauxic lag (Yoch and Pengra, 1966; Strandberg and Wilson, 1967). Addition of certain amino acids (especially aspartate) stimulated enzyme formation in the absence of ammonia in *K. pneumoniae* possibly by providing preformed organic nitrogen for enzyme synthesis. As in *K. pneumoniae*, nitrogenase synthesis in *A. chroococcum* was not repressed by the presence of aspartate, glutamate or glutamine nor were these compounds metabolized (Drozd *et al.*, 1972). Even during active nitrogen fixation, synthesis of nitrogenase was partially repressed by the intracellular pools of ammonia accumulated under these conditions. In ammonia-limited chemostat cultures therefore (with presumably much lower pool levels of ammonia) both *Cl. pasteurianum* and *Azotobacter chroococcum*, growing in the presence of an inert gas phase (but lacking nitrogen), contained higher nitrogenase activities than did nitrogen-fixing populations (Dalton and Postgate, 1969). Munson and Burris (1969) obtained similar results with fixed-nitrogen-limited chemostat cultures of the photosynthetic bacterium *R. rubrum*. In further experiments with *A. chroococcum* (Hill *et al.*, 1972), the nitrogenase activity of sulphate-limited chemostat cultures growing on molecular nitrogen decreased as the content of ammonia in the input medium was increased. Free ammonia could be detected in the medium only when nitrogenase synthesis was totally repressed. It was possible to obtain stable populations at different states of repression which suggested that nitrogen fixation and utilization of exogenous ammonia occurred at the same time. The degree of repression by a particular concentration of ammonia was a function of the culture population density and, in populations of low cell density, only low concentrations of ammonia were required to repress nitrogenase synthesis. This is of significance in natural environments in which only small populations of cells are normally detected. Furthermore, the addition of ammonia to nitrogen-fixing chemostat cultures of *A. chroococcum* was shown to curtail the culture nitrogenase activity at an exponential rate; the culture enzyme activity therefore decreased faster than predicted for the wash-out of stable enzyme (Drozd *et al.*, 1972). This indicated that, as in *A. vinelandii* (Hardy *et al.*, 1968) ammonia had a double effect, bringing about repression of nitrogenase synthesis and, concomitantly, a small decrease in activity of existing nitrogenase. In *Cl. pasteurianum* and *K. pneumoniae*, the effect of ammonia may be solely one of repression since preformed nitrogenase remained active in the presence of ammonia (Daesch and Mortenson, 1972; Mahl and Wilson, 1968). In *A. vinelandii* the synthesis of both of the constituent proteins of nitrogenase was repressed co-ordinately in the presence of ammonia and derepressed in its absence (Shah *et al.*, 1972).