THE EFFECT OF ADDED NH_4^+ IONS AND O_2-SUPPLY ON THE N-METABOLISM OF *AZOTOBACTER CHROOCOCCUM* IN CONTINUOUS CULTURE UNDER INTENSE AGITATION

BY

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A Thesis

submitted to

the Faculty of Graduate Studies and Research

UNIVERSITY OF MANITOBA

In partial fulfillment

of the requirements for the degree of

MASTER OF SCIENCE

AUGUST, 1976

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Professor H. Lees, my advisor, for his direction, criticism and assistance of both a financial and a conceptual nature throughout the course of this investigation and in the preparation of this thesis.

Special thanks are extended to the staff and graduate students of the Department of Microbiology, particularly to Y. K. Chan, A. Buchanan, D. Dawson and S. Byrne for their help and stimulating discussions.

I also thank my sisters Winnie and Christine for typing and drawing the figures.

TO MY PARENTS

AND

SYLVIA

ABSTRACT

Azotobacter chroococcum ATCC 7493 has been grown in continuous cultures at vortex agitation rate (1750 r.p.m.) under N_2-fixing condition and in the presence of various concentrations of NH_4^+. At the same time the O_2 concentration in the gas supply has been varied between 5 and 30%. The cultures were allowed to reach a steady-state (stabilized condition) for three days and the bacteria were then measured for their respiratory activity and analyzed for the content of various enzymes (nitrogenase, GOGAT, GLNS and GDH). The cultures, so stabilized, showed growth characteristics and enzyme patterns not entirely in harmony with the results of previous short term experiments. In particular nitrogenase activity was not completely suppressed even by high concentration of NH_4^+ (20 mM). The nitrogenase activity of the organism was always higher at low O_2 concentration than at high concentration of O_2 irrespective of whether NH_4^+ was present or not, and irrespective of the respiratory index of the organism. Moreover, in these long terms experiments NH_4^+-growing cells proved to be less, not more, tolerant of O_2 than were N_2-fixing cells.

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LIST OF ABBREVIATIONS

CPS	carbamyl phosphate synthetase.
GDH	glutamate dehydrogenase.
GLNS	glutamine synthetase.
GOGAT	glutamine(amide):2-oxoglutarate amino transferase oxido-reductase (NADP), also known as glutamate synthetase or glutamate synthase.
R.I.	Respiratory Index, see page 77.

INTRODUCTION

INTRODUCTION

It has been generally accepted for a long time that the nitrogenase activity of the nitrogen-fixing microorganisms is lowered when the organisms are supplied with inorganic nitrogen compounds particularly NH_4^+ and various theories e.g. the repression of nitrogenase synthesis or a direct inhibition of the nitrogenase itself by the NH_4^+ ion have been advanced to explain the undoubtedly correct results of short-term experiments in this field. However, it should be noted that the great majority of such experiments have been indeed short-term experiments and have not provided any information about how a population of N_2-fixing organisms settles down to a steady-state condition when exposed to NH_4^+ for a lengthy period. It therefore seemed desirable to investigate the long term effect of NH_4^+ on the N-metabolism of a well known N_2-fixer especially since Gordon & Brill, 1972 have shown that Azotobacter vinelandii is able to produce mutants that retain their N_2-fixing abilities even when grown in the presence of approximately 30 mM NH_4^+ for several days.

Since we have now devised in this laboratory a technique for growing A. chroococcum under high aeration (high agitation) rates where the organisms reach high densities without O_2-limitation although the dissolved O_2 is zero or close to zero

(Hine, 1975 and Hine & Lees, 1976), by this technique, we desired to investigate the long term effect of NH_4^+ ions on the enzyme pattern of A. chroococcum subjected to various concentrations of NH_4^+ in the medium and to various concentrations of O_2 in the gas phase.

Such experiments, we felt, might provide results that would approximate to the response of Azotobacter to NH_4^+ ions under natural conditions e.g. to the response of Azotobacter in soil treated with ammonia-containing fertilizers. Whether this is true or not it nevertheless seemed desirable purely from the point of scientific interest to investigate the long term effect of NH_4^+ ions on the overall N-metabolism of A. chroococcum.

L I T E R A T U R E R E V I E W

LITERATURE REVIEW

The fascinating biological conversion of an inert gas N_2, into a more reactive form was thought about by people such as Priestley, Ingen-Housz and Davy at the turn of the nineteenth century (Burns & Hardy, 1975). However, no convincing evidence that such converison took place was obtained until 1888 when Hellriegel and Wilfarth proved to the skeptical scientific community that biological N_2-fixation did in fact exist (Burns & Hardy, 1975). Advancement in the understanding of the actual mechanism of N_2-fixation was very slow because of the insensitive assay methods available and also because of the lack of sufficiently pure cultures of N_2-fixing organisms to work with. Small wonder, that a proper understanding of N_2-fixation has come from active research carried out during the last 16 years. Parenthetically, it is interesting to note that one by-product of the researches in N_2-fixation, the Lineweaver-Burk Plot, has become extremely important in the development of all enzyme kinetics. It has been found that many procaryotic organisms like Azotobacter spp., Clostridium and many blue-green bacteria are able to fix N_2; the work described in this thesis is confined to Azotobacter chroococcum.

CHARACTERISTICS OF AZOTOBACTER SPECIES

Azotobacter spp. are widely distributed throughout the world (Jensen, 1954). All are large rods varying from 2.0 - 7.0 x 1.0 - 2.5 μm.. They occur singly, in pairs or irregular clumps and rarely in chains of more than four cells. Young Azotobacter cells with the exception of A. beijerinckii, are motile and have peritrichous flagella. When the cells age they lose their motility, shorten and assume an almost coccoid form. They do not produce endospores but form metabolically dormant cysts, a characteristic which distinguishes Azotobacter from other genera (Azomonas, Beijerinckia and Derxia) in the Azotobacteraceae family (Bergey's Manual 8th ed., 1974; Sadoff, 1975). Old cells of A. chroococcum are characterized by a water-insoluble brown pigment which in some strains becomes black, whereas A. beijerinckii cells turn yellow or cinnamon when old. A. vinelandii and A. paspali both produce water-soluble green pigments at all stages of growth.

THE BREAKTHROUGHS IN STUDIES OF NITROGEN FIXATION

The elucidation of the process mechanism whereby microorganisms are able to "fix" atmospheric N_2 has proved to be a most recalcitrant problem. In fact, the solution defied the best research efforts until the early 1960's. The first breakthrough was the successful application of anaerobic techniques to isolate an active cell-free N_2-fixing extract

from Clostridium pasteurianum by Carnahan et al. in 1960. Their classical work has opened the door to the present understanding of the nitrogenase enzyme complexes, and of the mechanism of the process of N_2-fixation. Since then, active nitrogenase complexes have been prepared from Rhodospirillum rubrum (Schneider et al., 1960), Bacillus polymyxa (Grau & Wilson, 1962), Azotobacter vinelandii (Bulen et al., 1964), Azotobacter chroococcum (Kelly, 1969a), Klebsiella pneumoniae (Eady & Postgate, 1972), Rhizobium bacteroids (Bergerson & Turner, 1970) and many other organisms.

The active extract of C. pasteurianum prepared by Carnahan et al. was oxygen-sensitive, cold-labile and required large quantities of pyruvate to reduce nitrogen to ammonia, now believed to be the first free product of N_2-fixation. Subsequent studies revealed a dual role for pyruvic acid: providing electrons to reduce ferredoxin through the oxidation of pyruvate and providing ATP from the acetyl phosphate formed during pyruvate oxidation (McNary & Burris, 1962; Hardy & D'Eustachio, 1964; Mortenson, 1964). The work with Clostridium stimulated the study of N_2-fixation in Azotobacter by Bulen et al. in 1965 and 1966. They found that an artifical reductant (dithionite) could replace the reduced ferredoxin, and ATP could be supplied in the form of an ATP generator such as the creatine-phosphate/creatine-kinase reaction. Since then supplementation of cell extracts with dithionite and a creatine-phosphate/creatine-kinase ATP generating

system has become a fairly standard reaction mixture for *in vitro* assays of nitrogenase activities.

The use of dithionite revealed the activity of an ATP-dependent hydrogenase associated with the nitrogenase complexes (Bulen *et al.*, 1965; Hardy *et al.*, 1965). This ATP-dependent hydrogenase is different from the conventional hydrogenases since its activity is not inhibited by carbon monoxide (Bulen, 1965; Burns & Bulen, 1965). However, *C. pasteurianum* and *K. pneumoniae* generate hydrogen as a product of anaerobic growth, but not Azotobacter spp.. Nevertheless, the universal association between the ability of the nitrogenase to fix N_2 and the possession of hydrogenase activity led Postgate (1974) to suggest that the hydrogenase must play some intimate role in N_2-fixation.

That isolated nitrogenase had a broad substrate specificity was quickly recognized (Hardy *et al.*, 1968; Postgate, 1970). Nitrogenases catalyze the reduction of numerous compounds containing triple bonds in addition to N_2. These include C_2H_2, CN^-, N_3^-, CH_3CN and analogous compounds. The H^+ is also reduced to H_2 in the absence of other electron acceptors. The reduction of C_2H_2 became the basis of the well known acetylene-reduction assay which is now used as a routine measurement of nitrogenase activity. In this assay, C_2H_2 is reduced by the nitrogenase to C_2H_4 which is rapidly detected by gas chromatography.

This assay is not only 10^3 times more sensitive than the assay that uses ^{15}N (detectable only in a mass spectrograph) but is a great deal simpler to carry out. The reduction of C_2H_2 to C_2H_4 is a two electron process and since NH_4^+ is a six electron reduction product it is therefore possible to get an estimate of actual N_2-fixing activities of nitrogenase simply by dividing the C_2H_2 reduction rate by 3 (Postgate, 1971).

NITROGENASE PROTEINS

Nitrogenases from different microorganisms have been fractionated, purified and characterized. It is interesting that they are remarkably similar in structure and composition. They can all be separated into two components, one containing molybdenum, iron and acid-labile sulphur called Mo-Fe protein (also called molybdoferredoxin, component I, K_p 1 or azofermo) and the other containing iron and labile sulphur, the Fe-protein (also called azoferredoxin, component II, K_p 2 or azofer).

Mo-Fe proteins of Klebsiella and Clostridium are tetrameric and can be dissociated into two different types of subunit of 50,000 and 60,000 daltons (Eady et al., 1972; Nakos & Mortenson, 1971b; Huang et al., 1973; Tso, 1974). Azotobacter Mo-Fe protein apparantly has only one type of subunit of 56,000 daltons (Kleiner & Chen, 1974).

From their subunit compositions, the Mo-Fe proteins of Klebsiella and Clostridium have been calculated to be 218,000 daltons (Eady et al., 1972) and 220,000 daltons (Huang et al., 1973) respectively; while that of Azotobacter is around 216,000 daltons (Kleiner & Chen, 1974).

Chen et al. (1973) studied the amino acid compositions of Mo-Fe proteins from Azotobacter, Klebsiella and Clostridium. The number of comparable amino acid residues among these three bacteria is strikingly similar except for the tryptophan content, with the Clostridium Mo-Fe protein having a low number of tryptophan residues compared with the other two.

Azotobacter Mo-Fe protein contains 1.54 Mo atoms, 24 Fe atoms and 20 acid-labile sulfide molecule (Kleiner & Chen, 1974). Klebsiella Mo-Fe protein contains about 1.2 Mo atoms, 17 Fe atoms and equal number of sulfides per 218,000 daltons (Eady et al., 1972). Clostridial Mo-Fe protein seems to have 2 Mo atoms, 12 - 18 Fe atoms and 8 - 15 atoms of acid-labile sulfides per molecule of 210,000 (Tso, 1974).

The role of Mo in Mo-Fe proteins is obscure (McKenna et al., 1970; Burns et al., 1971; Benemann et al., 1972; and Benemann et al., 1973). The results of a recent report (Nagatani & Brill, 1974) indicated that Mo was a possible inducer of Mo-Fe protein synthesis. Although tungsten would replace Mo in Mo-Fe protein this substitution rendered the

protein inactive. When A. vinelandii was derepressed in NH_4^+-free medium with vanadium, there were no recognizable vanadium-containing proteins formed.

The Fe-proteins from C. pasteurianum, 56,000 daltons (Nakos & Mortenson, 1971a; Tso, 1974) and K. pneumoniae, 66,800 daltons (Eady et al., 1972) are isolated as dimeric proteins with identical subunits. The Fe-protein of A. vinelandii also occurs in two equivalent subunits of 33,000 daltons each (Kleiner & Chen, 1974). The amino acid compositions of these bacteria have been determined (Eady et al., 1972; Chen, 1973 and Kleiner & Chen, 1974). The Azotobacter Fe-protein has an amino acid composition not quite the same as that of Klebsiella and Clostridium, but there is a common feature for all of them: they lack tryptophan.

The Fe-proteins from both Klebsiella and Clostridium contain 4 Fe atoms and 4 acid labile sulfides (Eady et al., 1972 and Tso, 1974); whereas that of Azotobacter contains 3.45 Fe atoms and 2.85 labile sulfides per molecule (Kleiner & Chen, 1974).

THE EFFECT OF OXYGEN

The effect of oxygen on the growth of many aerobic or facultative anaerobic bacteria, including Azotobacter has been studied since 1911 (Moore & Williams, 1911; Meyerhof &

Burk, 1928). Prior to the extensive researches by Postgate and his colleagues (Dalton & Postgate, 1969a,b; Drozd & Postgate, 1970a,b; Hill et al., 1972; Lees & Postgate, 1973) most of the work was done in batch cultures and therefore, intrinsically less appealing. The general ideas about the effect of oxygen can by summarized in the following:

(a) The growth of N_2-fixing Azotobacter is inhibited by a high pO_2 (Meyerhof & Burk, 1928; Burk, 1930; Dilworth & Parker, 1961).

(b) No O_2 inhibition, when A. chroococcum is assimilating bound nitrogen (Schmidt-Lorenz & Rippel-Baldes, 1957).

(c) The efficiency of N_2-fixation in A. vinelandii can be increased at a low pO_2 (Parker, 1954; Philips & Johnson, 1961; Khmel et al., 1965; Khmel & Gabinskaya, 1965).

(d) Some enzymes like pyruvic oxidase, acetate kinase and malate synthetase in Azotobacter can be damaged by high pO_2 (Dilworth, 1962; Dilworth & Kennedy, 1963).

Postgate and his colleagues examined in great detail the effect of oxygen on the growth of A. chroococcum in batch and continuous cultures. Their results indicated that:

(a) O_2 inhibition was specific to N_2-fixing cells, and NH_4^+-grown cultures did not show any appreciable sensitivity to O_2 (Dalton & Postgate, 1969a).

(b) The N_2-fixation effeciency was higher at a pO_2 lower than 0.2 atmosphere (Dalton & Postgate, 1969a).

(c) The degree of sensitivity of N_2-fixation towards O_2 inhibition was dependent on the nutritional status of the culture. Carbon-limited and phosphate-limited cells were particularly oxygen sensitive (Dalton & Postgate, 1969a; Lees & Postgate, 1973).

(d) Inhibition of C_2H_2 reduction by oxygen was reversible after mild oxygenation (Dalton & Postgate, 1969a; Drozd & Postgate, 1970).

To explain the reversible inhibition of nitrogenase activity by mild oxygenation and the observation that crude Azotobacter nitrogenase extracts are stable in air but not when purified, Postgate and his colleagues suggested two mechanisms for protecting Azotobacter nitrogenase from O_2. They are, namely, conformational protection and respiratory protection.

The conformational protection assumes that the steric arrangement of the nitrogenase components could operate in either making the oxygen sensitive site inaccessible to O_2 or rendering it undamaged even though accessible to O_2 (Dalton & Postgate, 1969a). Evidence that nitrogenase proteins undergo a conformational change were obtained by Yates (1972) who showed that the Fe-protein of A. chroococcum in the presence of ATP could change its conformation to become more O_2 sensitive. The same results were obtained in K. pneumoniae Fe-proteins (Thorneley & Eady, 1973).

Respiratory protection is thought to operate in a way such that respiration is used to scavenge O_2 from the neighborhood of the N_2-fixing sites. Actually this mechanism was first proposed by Phillips & Johnson (1961) as a result of their observation that A. vinelandii consumed more sugar than necessary when given excess O_2.

While both protein components of nitrogenase are made O_2 sensitive, the Fe-protein is by far the more labile. The degree of sensitivity increases with pO_2 and with temperature, and also varies between different organisms and between different methods of preparation (D'Eustachio & Hardy, 1964; Kelly, 1969b; Yates, 1970).

O_2 was once thought to be a competitive inhibitor of N_2-fixation (Parker & Scutt, 1958 & 1960). These authors suggested that O_2 and N_2 might compete as alternative acceptors of respiration electrons and regarded N_2-fixation as a form of respiration. However, recently, Wong & Burris (1972) showed unambiguously that O_2 inhibited the nitrogenase of A. vinelandii from reducing N_2, acetylene, azide and cyanide uncompetitively. It was also found that O_2 did not affect the electron transport from ascorbate to Azotobacter flavoprotein through dichlorophenolindophenol and illuminated spinach chloroplasts. However, O_2 did inhibit the nitrogenase dependent ATP hydrolysis.

It has been reported that O_2^-, the superoxide anion is readily generated by many spontaneous and enzymatic oxidations (Fridovich, 1974). The powerful reactivities of the superoxide anions can cause oxidations of epinephrine, catechols, dehydrogenase-bound NADH and also reduction of cytochrome c and Fe (III) in ferritin (Fridovich, 1975). The reaction of O_2^- with H_2O_2 to produce the hydroxyl radical (OH.) and singlet O_2*

$$H_2O_2 + O_2^- \longrightarrow OH^- + OH. + O_2*$$

the extremely powerful oxidants (Kellogg & Fridovich, 1975), may further enhance its potential dangers. Fortunately, an O_2 inducible enzyme, superoxide dismutase that can scavenge O_2^- is wide-spread among aerobic organisms (McCord et al., 1971), and this enzyme undoubtedly plays an important role in protecting aerobic organisms from O_2 poisoning. It would be interesting to study if this enzyme is present in aerobic N_2-fixers and its possible role in protecting the nitrogenase.

THERMODYNAMICS OF NITROGEN FIXATION AND ATP REQUIREMENTS

The thermodynamic aspects of N_2-fixation were first considered by Wilson & Burris (1947), and then critically re-examined by Bayliss (1956) who compared the free energy changes in the following reactions:

$$C_6H_{12}O_{6(aq)} + 4\ N_{2(gas)} + 6\ H_2O_{(liq)} + 8\ H^+_{(aq)} \longrightarrow 6\ CO_{2(gas)} + 8\ NH_4^+{}_{(aq)} \qquad \Delta G = -112\ Kcal \qquad \text{Eqn 1}$$

$$C_6H_{12}O_{6(aq)} + 6\ O_{2(gas)} \longrightarrow 6\ CO_{2(gas)} + 6\ H_2O_{(liq)} \qquad \Delta G = -690\ Kcal \qquad \text{Eqn 2}$$

The exothermic and exergonic nature of Eqn 1 led Bayliss to the conclusion that the N_2-fixation process, if occurred according to Eqn 1, could certainly require no assistance from any other energy providing reactions. However, it is not known whether the reaction actually occurs in this manner, nor is it known whether the released energy could be utilized in other biological reactions.

Bergersen (1971) re-examined the difference in energy yield when glucose was oxidized by oxygen or nitrogen. By substracting Eqn 2 from Eqn 1, he obtained

$$4\ N_{2(gas)} + 12\ H_2O_{(liq)} + 8\ H^+_{(aq)} \longrightarrow 8\ NH_4^+{}_{(aq)} + 6\ O_2 \qquad \Delta G = +578\ Kcal \qquad \text{Eqn 3}$$

Again, Eqn 3 could be expressed as two partial reactions:

$$4\ N_{2(gas)} + 12\ H_{2(gas)} + 8\ H^+_{(aq)} \longrightarrow 8\ NH_4^+{}_{(aq)} \qquad \Delta G = -174\ Kcal \qquad \text{Eqn 4}$$

and $12\ H_2O_{(liq)} \longrightarrow 12\ H_{2(gas)} + 6\ O_{2(gas)}$

(by difference) $\Delta G = +752$ Kcal Eqn 5

Bergersen's equations, derived from that of Wilson & Burris (1947) and Bayliss (1956) indicated that a net input of about 72 Kcal of metabolic energy per mole NH_4^+ would be required to drive the N_2 reducing reaction, and this would be due to the metabolic provision of reducing power at the level of hydrogen.

Unfortunately, neither *in vitro* nor *in vivo* studies give an unequivocal answer to the question: How much ATP is necessary for N_2-fixation? Dalton & Postgate (1969b) showed in *A. chroococcum* a maximum requirement of 4 moles ATP/mole N_2 fixed (equivalent to 1.3 ATP/$2e^-$ or 2 ATP/NH_4^+). This figure is low compared to that for Klebsiella and Clostridium which fall between 18 - 33 moles ATP/N_2 (Hill, 1972). However Dalton & Postgate's calculation was based on the Y_{ATP} value of 10.5 from Bauchop & Elsden (1960).

Recently, Watt and his co-researchers (Watt *et al.*, 1975) studied the energy requirements for reactions catalyzed by nitrogenase from *A. vinelandii*. The ATP/$2e^-$ ratio was found to go through a minimum of 4.2 at 20° C over a range from 10° to 35° C. The authors proposed that the Fe-protein of the nitrogenase could be reversibly converted into inactive form below 20° C, and reconversion of the protein into active

form could be brought about by raising the temperature or by increasing the ATP hydrolysis. The increase of ATP/$2e^-$ above 20^o C was thought to be the "uncoupling" of the nitrogenase components.

Bui & Mortenson (1968) showed that ATP and ADP were bound to Fe-protein but not to Mo-Fe protein in Clostridium nitrogenase components. The binding of ATP required Mg^{++} in the form of $Mg\text{-}ATP^{--}$, but not ADP. In each case, about 0.4 mole of nucleotide was bound per mole Fe-protein. In the Klebsiella nitrogenase system, $Mg\text{-}ATP^{--}$ was bound to both Mo-Fe protein and Fe-protein, and the ratio of $Mg\text{-}ATP^{--}$ to Fe-protein was 0.6 (Biggins & Kelly, 1970). Azotobacter Fe-protein was also known to bind ATP in the presence of Mg^{++} (Yates, 1972).

Although ATP is required in N_2-fixation, the mechanism of its interaction with nitrogenase remains obscure. The products of ATP hydrolysis are ADP and inorganic phosphate; no additional products arising from ATP have been identified (Hardy & Knight, 1966; Kennedy et al., 1968). Other nucleotide triphosphates cannot substitute for ATP (Moustafa & Mortenson, 1967; Hardy et al., 1968). The binding of ATP to nitrogenase might change the ligand conformation around a transitional metal, thus creating an active species for the reduction of substrate (Silverstein & Bulen, 1970; Yates, 1972; Zumft et al., 1972 and Zumft et al., 1973).

ATP might also be used as electron activator in the N_2-fixing reaction (Hardy & Burns, 1968).

REGULATION OF THE ACTIVITIES OF THE NITROGENASE

N_2-fixation is a very complex reaction; undoubtedly it is affected by the overall physiology of the organism. So far, the activity of nitrogenase has been reported to be regulated by O_2, ADP and ATP concentrations, and by carbamyl phosphate.

The "switch-on and switch-off" of nitrogenase refers to the reversible inhibition of nitrogenase by oxygenation, and has been discussed under the heading of " The Effect of Oxygen"(see page 11).

The ADP regulates the activity of nitrogenase by binding to the Fe-protein (Moustafa & Mortenson, 1967; Kennedy, 1970). The Fe-protein contains two Mg-ATP^{--} binding sites, one of which also binds ADP (Tso & Burris, 1973). The authors found the 'dissociation constant' of ATP at either of two sites to be 17 μM and that of ADP to be about 5 μM. Another ADP binding site, found by Zumft et al. (1974) can bind ADP at a much higher dissociation constant. The binding of ADP inhibits all reactions catalyzed by nitrogenase; probably its binding prevents Mg-ATP^{--} from being consumed and electrons from being transferred to Mo-Fe protein.

Seto & Mortenson (1973 & 1974a) demonstrated that carbamyl phosphate, a product of early ammonia incorporation, could inhibit C_2H_2 reduction, N_2-fixation and the biosynthesis of nitrogenase. However, it has no effect on the reductant-dependent ATP hydrolysis and H_2 evolution reaction. Unlike ADP, carbamyl phosphate binds to Mo-Fe protein rather than to Fe-protein, and an optimal binding is obtained only in the presence of an operative nitrogenase system. Seto & Mortenson (1974a) observed two carbamyl phosphate bindings per Mo-Fe protein tetramer and that the dissociation constant was about 50 - 70 μM. Carbamyl phosphate could be a potentially significant tool in the elucidation of the mechanism of nitrogenase because of its differential effect on nitrogenase activities and because unlike CO, it is not a gas.

CONTROL OF NITROGENASE BIOSYNTHESIS

The idea that N_2 is an inducer of nitrogenase synthesis is highly questionable. Investigations using Azotobacter (Strandbery & Wilson, 1968) and Klebsiella (Parejko & Wilson, 1972) did not give decisive results because N_2 was present in trace amount with the oxygen and helium used. Nevertheless, Mortenson (1968) concluded that it was very unlikely that the organism would possess a system capable of responding to such a low concentration of metabolizable substrate - particularly since such an environment was most unlikely in nature and no selective pressure for such a control would exist.

The effect of NH_4^+ on nitrogenase has been extensively studied in various microorganisms. It is now commonly assumed that high level of NH_4^+ acts as repressor or co-repressor of nitrogenase synthesis, whereas under some circumstances very low levels are able to stimulate synthesis of the enzyme (Burris, 1971). Kleiner (1974) observed that there was no repression of A. vinelandii nitrogenase below 10 μM NH_4^+, whereas repression was completed if the NH_4^+ concentration in the medium exceeded 3.6 mM.

It is interesting to note that although extracellular NH_4^+ above 10 μM appears to be repressive to the nitrogenase system, many N_2-fixing organisms maintain a high NH_4^+ concentration gradient across the cell membrane. When fixing N_2, K. pneumoniae (Yoch & Pengra, 1966) and A. chroococcum (Drozd et al., 1972) accummulate high level of intracellular NH_4^+, up to 3 and 2 mM respectively. The reasons and mechanisms for keeping such high level of NH_4^+ and yet not repressing the nitrogenase to a large extent are unknown.

Tubb & Postgate (1973) found the half-life of Klebsiella nitrogenase in chemostat culture after the addition of NH_4^+ (95 min.) was shorter than the theoretical half-life of washing out (288 min.). When chloramphenicol was added in addition to NH_4^+, the nitrogenase half-life increased to 240 min., suggesting that protein synthesis might be required for the inactivation. Davis et al.(1972) observed that in

A. vinelandii during repression, the immunochemically detectable nitrogenase protein decayed inversely with cell growth, while the activity and EPR signals were depressed considerably more rapidly. The authors suggested there was a rapid mechanism of Mo-Fe protein inactivation, followed by a slower process in which the protein portion was finally broken down and reutilized. Strandbery & Wilson (1968) and Drozd et al. (1972) also observed in Azotobacter that the nitrogenase activity decreased faster than predicted if the enzymes simply diluted out. However Mulder & Brotonegoro (1974) argued that the rapid decline of nitrogenase activity in living Azotobacter supplied with NH_4^+ was not due to repression of nitrogenase synthesis or feedback inhibition but an effect of competition for reductants and/or ATP between nitrogenase activity and assimilation of NH_4^+.

Studies of nitrogenase synthesis with the mRNA inhibitor rifampicin and the protein synthesis inhibitor chloramphenicol show that Klebsiella requires 40 min. to reach maximum nitrogenase synthesis after NH_4^+ exhaustion (Tubb & Postgate, 1973), whereas Clostridium requires 60 min. (Seto & Mortenson, 1974b). In Clostridium, translation is initiated before the mRNAs are completed, a mechanism similar to that in E. coli (Seto & Mortenson, 1974b). Moreover, the observation of 15 min. difference between the time of the appearance of Mo-Fe protein and Fe-protein activies enabled the authors to conclude that the structural genes for the two nitrogenase

components might not be contiguous, or might not be linked, or might not have a similar operator. In contrast, the A. vinelandii shows co-ordinate synthesis of the two nitrogenase components (Shah et al., 1972).

Although NH_4^+ represses nitrogenase, it is not the actual effector for the repression. Evidence supporting this argument are furnished by Brill and his co-researchers. Gordon & Brill (1972) isolated some mutants of A. vinelandii which could fix N_2 in the presence of excess NH_4^+. They (Gordon & Brill, 1974) observed that methionine sulfone and methionine sulfoximine, inhibitors of the NH_4^+ assimilation enzymes glutamate synthase and glutamate dehydrogenase caused nitrogenase to be synthesized in the presence of excess NH_4^+ in A. vinelandii and K. pneumoniae. These inhibitors also caused NH_4^+ excretion by A. vinelandii. These results indicate that NH_4^+ per se is not the actual effector of nitrogenase repression in these organisms. Moreover, these results indicate the possibility of involving glutamine or glutamate or the enzymes of ammonia assimilation in regulation of nitrogenase biosynthesis.

Nitrogenase biosynthesis is at least partially regulated by the enzyme glutamine synthetase (Streicher et al., 1974). Mutation causing constitutive synthesis of glutamine synthetase ($GlnC^-$ phenotype) was transferred from K. aerogenes into K. pneumoniae by phage P1-mediated transduction.

The $GlnC^-$ phenotype in such obtained K. pneumoniae strains allowed the synthesis of nitrogenase to continue under conditions that completely repressed nitrogenase synthesis in the wild type strain. Moreover, Gln^- K. pneumoniae that did not produce catalytically active glutamine synthetase and consequently synthesized no nitrogenase could be complemented with an E. coli episome (haboring Gln^+) to restore the activities of both enzymes. It is therefore concluded that the deadenylylated glutamine synthetase functions as the nitrogenase gene activator, controlling its biosynthesis. Confirmation was obtained by Tubb (1974) when an F factor containing the nitrogenase genes of K. pneumoniae was transferred to a non-N_2-fixing strain of K. aerogenes having a mutation that caused formation of constitutive levels of glutamine synthetase. This hybrid strain synthesized high levels of nitrogenase in the presence of excess NH_4^+, thus supporting the conclusion that glutamine synthetase regulates the biosynthesis of nitrogenase. However, the mechanism has yet to be determined.

REACTIONS OF AMMONIUM ASSIMILATION

The behaviour of organisms in response to nitrogen availability follows the law of supply and demand. In order to safeguard a steady supply of nitrogen, a tight control over the ammonium assimilation enzymes is essential. Incorporation of NH_4^+ into organic materials is mainly carried out by the enzymes glutamate dehydrogenase (GDH),

glutamine synthetase (GLNS) and carbamyl phosphate synthetase (CPS) (Mahler & Cordes, 1971 p. 775). They respectively catalyze the following reactions:

$$\alpha\text{-ketoglutarate} + NH_4^+ + \begin{matrix}NADH\\NADPH\end{matrix} \xrightarrow{GDH} \text{L-glutamate} + H_2O + \begin{matrix}NAD^+\\NADP^+\end{matrix}$$

$$\text{L-glutamate} + NH_4^+ + ATP \xrightarrow{GLNS} \text{L-glutamine} + ADP + Pi$$

$$\text{L-glutamine} + 2\ ATP + HCO_3^- \xrightarrow{CPS} \text{L-glutamate} + 2\ ADP + Pi + NH_2\text{-}COOPO_3H_2$$

$$NH_3 + 2\ ATP + HCO_3^- \xrightarrow{CPS} 2\ ADP + Pi + NH_2\text{-}COOPO_3H_2$$

There are two types of carbamyl phosphate synthetase. The microbial enzymes use glutamine as nitrogen donor while the ammonium requiring enzymes have not been found in organisms lower than the earthworm in the evolutionary scale.

Recently Meers and his co-researchers (Meers et al., 1970a,b; Tempest et al., 1970a) found in K. (Aerobacter) aerogenes that glutamate was synthesized by a reaction coupled in the GLNS catalyzed formation of glutamine. This novel reaction is catalyzed by an enzyme glutamate synthase, also known as glutamine (amide):2-oxoglutarate amino transferase oxido-reductase(NADP) or GOGAT. The reaction is:

$$\alpha\text{-ketoglutarate} + \text{L-glutamine} + NADPH \xrightarrow{GOGAT} 2\ \text{L-glutamate} + NADP^+$$

This enzyme GOGAT has been found in wide variety of procaryotic organisms including the aerobic N_2-fixer Azotobacter (Nagatani et al.,1971) and the anaerobe C. pasteurianum (Dainty, 1972).

GLUTAMATE DEHYDROGENASE (GDH)

The GDH enzymes isolated from various sources are widely different in terms of kinetic characteristics, metabolic functions and molecular properties. Generally, animal enzymes are sensitive to purine nucleotides, can use either NAD or NADP, and frequently undergo reversible polymerization reaction. However the non-animal enzymes are not affected strongly by purine nucleotides, do not undergo reversible polymerization, and are specific for either NAD or NADP (Goldin & Friedin, 1971 and Smith et al., 1975). A variety of organisms like Neurospora (Sanwal & Lata, 1961), Thiobacillus (LeJohn & McCrea, 1968) and pseudomonads (Brown et al., 1973) are capable of synthesizing both NAD and NADP dependent GDHs, while others like E. coli (Halpern & Umbarger, 1960) and Clostridium SB_4 (Winnocker & Barker, 1970) synthesize NADP and NAD dependent enzymes respectively.

The Clostridium SB_4 enzyme has a molecular wt. of 275,000 (Winnocker & Barker, 1970). The GDH from E. coli has a molecular wt. of 300,000 and when dissociated the enzyme gives six subunits with identical molecular wts. of

50,000. The E. coli GDH is very stable at 4° C and room temperature but becomes inactivated by freezing and thawing (Sakamoto et al., 1975). All the GDHs are highly specific for their substrates. The E. coli GDH has Michaelis constants 1,100, 640 and 40 μM for NH_4^+, α-ketoglutarate and NADPH respectively (Sakamoto et al., 1975).

The studies on those organisms synthesizing both NAD and NADP-dependent GDHs have revealed their separate role in catabolism and biosynthesis. The NAD-dependent GDH functions in the catabolism of glutamate while the NADP-dependent enzyme is responsible for ammonium incorporation (Sanwal & Lata, 1962; Brown et al., 1973). The studies on bacteria that produce only one form of GDH also support this concept of coenzyme specificity and metabolic role (Goldin & Frieden, 1971). Thus, Clostridium SB_4 uses the NAD-dependent GDH to produce α-ketoglutarate for fermentation purposes (Winnocker & Barker, 1970) and the NADP-dependent GDH of E. coli mediates the synthesis of glutamate (Halpern & Umbarger, 1960). However, the Km values of NADP-dependent GDHs towards NH_4^+ are generally high (Miller & Stadtman, 1972 and Smith et al., 1975) and it appears unlikely that they can function efficiently in NH_4^+ assimilation, except when the environmental ammonium concentration is high.

GLUTAMATE SYNTHASE (GOGAT)

Miller and Stadtman (1972) purified the GOGAT from E. coli to homogeneity. Disaggregation studies showed the enzyme might be composed of 8 subunits, 4 each of 2 dissimilar types of molecular wts. 135,000 and 53,000. Each enzyme molecule might contain 32 iron atoms, 32 labile sulfide atoms and 8 non-covalently bound flavin molecules.

This enzyme is highly specific for substrate requirements. In K. (Aerobacter) aerogenes (Meers et al., 1970b) and E. coli (Miller & Stadtman, 1972) NADH cannot replace NADPH, nor can pyruvate, oxaloacetate, 2-oxobutyrate and 2-oxoisovalerate replace α-ketoglutarate. The requirement for L-glutamine also cannot be met by asparagine, citrulline, argenine, urea, NH_4Cl, D-glutamine or alkylated glutamine analogues. Recently, it was found that the GOGAT in K. (Aerobacter) aerogenes (Trotta et al.,1974) and in E. coli K12 (Mäntsälä & Zalkin, 1976a,b) were able to use NH_3 in addition to L-glutamate. However, the NH_3-dependent GOGAT activity was very low, about 5 - 7% of the glutamate-dependent GOGAT activity. The GOGAT enzymes from various organisms show widely different Km values for L-glutamine and α-ketoglutarate (Meers et al., 1970b). For example, the Klebsiella and E. coli enzymes have apparent Km values 1,800 and 250 μM for L-glutamine and those for α-ketoglutarate are 2,000 and 7.3 μM respectively. Inhibition studies with E. coli (Miller & Stadtman,1972) showed that only

NADP, D & L-aspartate, D-glutamine and L-methionine produced 50% inhibition at concentrations less than 7 mM, but cyclic AMP, AMP and ADP had no effect upon the activity of the enzyme. These results may indicate that the GOGAT enzymes are subject to feedback inhibition in the highly divergent metabolic pathways leading from glutamate.

Inorganic ions show marked effects on the GOGAT activity. Enzymes from different organisms invariably have optimium activity at an alkaline pH of 7.5 - 8.5 (Meers et al., 1970b). The E. coli enzyme has an optimum pH of 7.6 (Miller & Stadtman, 1972). The Klebsiella enzyme is not appreciably affected by Na^+ and K^+, except when present in high concentration. The Mg^{++}, however, is a potent inhibitor. Similar results have been observed with the E. coli enzyme. Inhibitor divalent cations include Ba^{++}, Ca^{++}, Co^{++}, Cd^{++} and Ni^{++}. The latter three cations are able to produce more than 60% inhibition at a concentration of 5 mM. The anions Cl^-, Br^- and NO_3^- are modest inhibitors whereas acetate, citrate and SO_4^{--} have no significant effect upon activity (Miller & Stadtman, 1972).

The reaction catalyzed by GOGAT is irreversible (Nagatani et al., 1972; Miller & Stadtman,1972). This reaction when coupled with that catalyzed by GLNS gives

$$NH_4^+ + \alpha\text{-ketoglutarate} + ATP + NADPH \longrightarrow L\text{-glutamate} + ADP + Pi + NADP^+$$

which is almost identical with the reaction catalyzed by GDH, with the exception of the expenditure of one high energy bond from ATP. (Presummably this expenditure of energy is essential in order to assimilate ammonium into the amide group under conditions where it is present in very low concentration.)

GLUTAMINE SYNTHETASE (GLNS)

Because of the significance of glutamine in the branched metabolic pathways the enzyme catalyzing glutamine formation has been studied extensively, especially in E. coli (Stadtman et al., 1970; Prusiner & Stadtman, 1973). The E. coli GLNS has 12 subunits arranged in two hexagons facing each other. The enzyme molecule behaves like a spherical particle of 600,000 daltons and each subunit being 50,000 daltons.

The E. coli GLNS is subject to feedback inhibition by the multiple end-products of glutamine metabolism. At a physiological concentration, each of alanine,glycine, histidine, tryptophan, CTP, AMP, carbamyl phosphate and glucosamine-6-phosphate produce partial inhibition. However, mixtures of these inhibitors show cumulative effects. Thus, collectively they can cause almost complete inhibition of the enzyme activity (Woolfolk & Stadtman, 1967). Kinetic studies and binding studies (Woolfolk & Stadtman, 1967 and Ginsburg, 1969) suggest that the GLNS has separate sites for most of the inhibitors. The E. coli GLNS has very low Km value for NH_4^+

(<0.2 mM, Miller & Stadtman, 1972) and it has been proposed that the coupled reactions catalyzed by GLNS and GOGAT would be the major route to function at low level of NH_4^+.

The most intriguing control of the E. coli GLNS is the ability of the tyrosyl residues to be adenylylated by an enzyme adenylyl transferase to form a 5'-adenylyl-O-tyrosyl derivatives thus changing the activity and the properties of the enzyme. For instance, the formation of deadenylylated GLNS is activated by Mg^{++} or Co^{++} while that of adenylylated enzyme is activated by Mn^{++} (Kingdon & Stadtman, 1967 and Ginsburg & Stadtman, 1973).

The deadenylylated GLNS is considered to be active in vivo for the catalysis of glutamine formation, while the adenylylated enzyme is not. Evidence supporting this statement is based on the fact that almost completely adenylylated GLNS is present in cells grown in excess of nitrogen, whereas the enzyme present in cells grown under condition of nitrogen starvation is almost completely adenylylated (Kingdon & Stadtman, 1967).

Both adenylylated and deadenylylated GLNS, under proper assay conditions are able to transfer the glutamyl group of the glutamine to hydroxylamine in the presence of ADP as shown in the following reaction.

$$\text{L-glutamine} + NH_2OH \xrightarrow{\text{GLNS}} \gamma\text{-glutamylhydroxamate} + NH_3$$

However, the adenylylated GLNS can carry out this reaction in the presence of Mn^{++} but not in 60 mM $MgCl_2$. Hence, assaying the GLNS by the transferase activity in the presence of 0.3 mM $MnCl_2$ measures the total amount of both adenylylated and deadenylylated enzymes. Assaying the GLNS in the presence of both 0.3 mM $MnCl_2$ and 60 mM $MgCl_2$ gives only the measurement of the deadenylylated enzyme which correlates closely to the *in vivo* activity of the enzyme.

The regulation of GLNS by adenylylation and deadenylylation exists exclusively in Gram-negative bacteria (Wohlhueter *et al.*, 1973 and Tronick *et al.*, 1973). This was demonstrated by the ability of the GLNS from Gram-negative bacteria to cross react with antisera prepared against adenylylated and deadenylylated *E. coli* GLNS. Furthermore, GLNS from Gram-negative bacteria are sensitive to the treatment with snake venom phosphodiesterase which is known to remove the adenylyl groups (or other nucleotidylated subunits) from the *E. coli* GLNS. Hence, it is very likely that a regulatory mechanism similar to that of *E. coli* GLNS is operating in the Gram-negative bacteria. The GLNS of *A. vinelandii* and *A. agilis* behaved quite differently in the above mentioned immunological test with antisera prepared against *E. coli* enzyme. Precipitin bands were formed with the enzyme of *A. vinelandii* only in the presence to either $MgCl_2$ or $MnCl_2$, but none with those of *A. agilis*.

However, the γ-glutamyl transferase activity of A. agilis GLNS was strongly inhibited by the antibodies, indicating the recognition of the enzyme by the antibodies although no precipitin band was formed. The presence of an adenylylation/deadenylylation regulatory mechanism of the GLNS in Azotobacter was confirmed by the fact that the enzymes were highly sensitive to snake venom phosphodiesterase (Tronick et al., 1973).

PATHWAYS OF AMMONIUM ASSIMILATION

The relation between the three ammonium assimilation enzymes GDH, GLNS and GOGAT can be briefly shown in Fig. 1, the Glutamate Cycle. The operation of the Glutamate Cycle appears to be a highly sophisticated method for the bacteria to guarantee a steady supply of nitrogen regardless of the fluctuation of the N-source in the surroundings. In K. (Aerobacter) aerogenes (Meers et al., 1970b; Meers & Tempest, 1970) a reciprocal relation exists between the cellular contents of GOGAT and GDH. Thus, glucose-limitation favours the synthesis of GDH but not GOGAT/GLNS, and the reverse is true under NH_4^+-limitation. This finding supports the idea that the high Km values of GDH for NH_4^+ render the enzyme functionally inadequate at low level of NH_4^+, at which the GLNS takes over the job because of its lower Km.

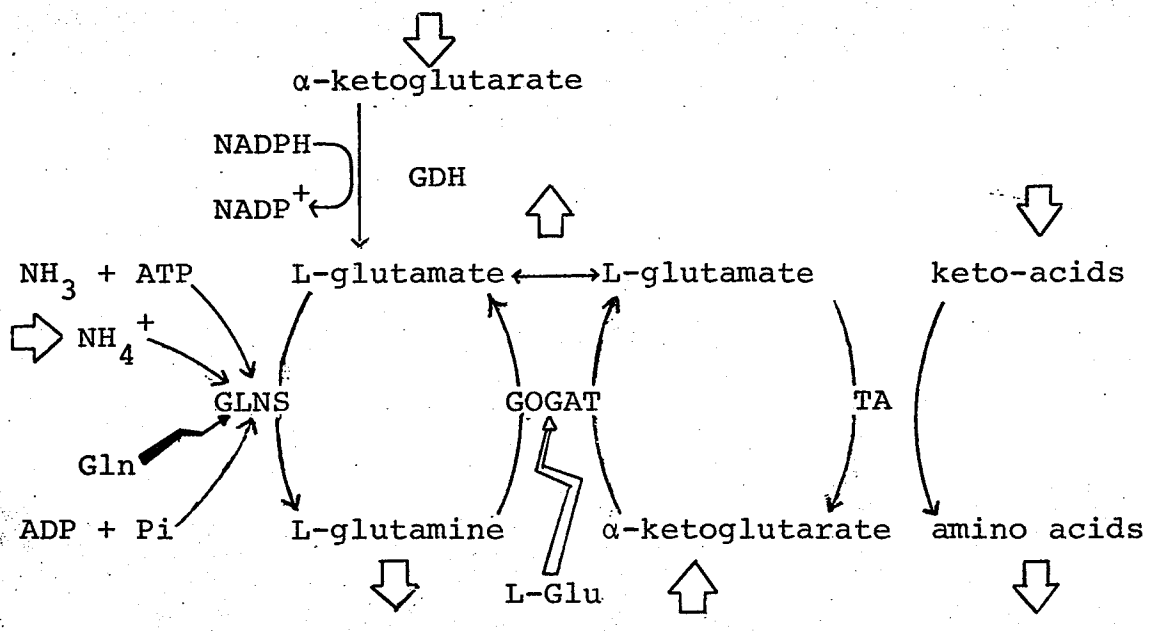


Fig. 1 The Glutamate Cycle, adapted from Tempest et al., 1970a and Elmerich, 1972.

Straight hollow arrows indicate entrance into and exits from the cycle.

The broken hollow arrow indicates the repression of biosynthesis of GOGAT by L-glutamate.

The broken black arrow indicates an allosteric inhibition.

Non standard abbreviations: GLNS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase and TA, transaminase.

Similar control is observed in some pseudomonads (Brown et al., 1972 & 1973). Bacteria like Erwinia carotovora (Meers et al., 1970b) and Bacillus megaterium (Elmerich, 1972) lacking GDH have to assimilate NH_4^+ via the GOGAT/GLNS couple. However in E. coli the control seems to be different from the K. aerogenes type. Senior (1975) followed the changes of the enzyme level of a E. coli chemostat culture growing at slow and fast rates with NH_4^+-limitation. At a slow specific growth

rate the GDH and GOGAT were present at low levels, but the GLNS was high and in a deadenylylated form. With increasing growth rate, the GDH increased linearly. Both the GLNS and GOGAT remained fairly constant until close to the maximum specific growth rate at which NH_4^+ was detectable and there was an abrupt decrease of GLNS and a rapid rise of GOGAT. However when K. aerogenes was grown in the same condition, the GDH did not appear and rise until close to the maximum growth rate, but the pattern of changes of GLNS and GOGAT was similar to that of E. coli . When glutamate was used as a limited N-source for E. coli the GOGAT was just barely detectable throughout slow and fast growth rates, but huge quantity of GLNS was synthesized. The GDH quickly rose to a plateau and fell. It appeared, therefore, that the E. coli GDH could play a biosynthetic role during NH_4^+-limitation and a degradative role during glutamate-limited growth.

CONTROL MECHANISMS OF THE AMMONIUM ASSIMILATION ENZYMES

No simple model is apparent for the co-ordinated control of these enzymes. In general, high level of NH_4^+ favours the synthesis of GDH but not GOGAT/GLNS; and the GLNS, if produced, is mainly in adenylylated form (Mecke & Holzer, 1965; Woolfolk et al., 1966; Meers et al., 1970b; Meers & Tempest, 1970; Brown et al., 1973; Prival et al., 1973; Senior, 1975). The reverse situation is true in N-limitation. The effect of L-glutamate seems to be to induce GLNS and to repress both

GDH and GOGAT; and GLNS is being repressed by L-glutamine (Wu & Yuan, 1968; Varricchio, 1969; Meers et al., 1970b; Elmerich, 1972; Senior, 1975).

Although it is quite easy to order the synthesis of the desired enzymes, there is no means of telling what is the actual effector responsible for the switching on and off of the enzymes. Meers & Tempest (1970) observed the normal expected changes of GDH, GOGAT and GLNS in K. (Aerobacter) aerogenes growing under glucose and NH_4^+ limitation. However the addition of 2% (w/v) of NaCl to the glucose-limited and the NH_4^+-limited cultures greatly increased the intracellular free glutamate concentration but had little effect in the content of GLNS. The use of L-glutamate as a N-limited component did not alter the intracellular glutamate level significantly, yet the GOGAT and GDH were totally repressed. Therefore the authors concluded that glutamate per se was neither the inducer of GLNS nor the repressor of both GDH and GOGAT.

Magasanik and his co-researchers (Brenchley et al., 1973) working on K. aerogenes isolated some glutamate requiring mutants (MK 204 and MK 208) which had lost the ability to use ammonium (33mM) as the sole N-source. These mutants produced tremendous amount of GLNS but very little GDH, no matter when glutamate is supplied in excess or in growth limiting rate. Furthermore, another glutamate requiring mutant (MK 71) was

able to maintain the GDH, GOGAT and GLNS levels the same as the wild type growing in excess of NH_4^+, whether glutamate was provided in excess or as a growth limiting nutrilite. These results support Tempest's idea that glutamate does not affect the production of the NH_4^+ assimilation enzymes.

The most interesting thing Magasanik and his group did (Brenchley et al., 1973) was to transduce the GLNS gene from donor (MK 204) which lacked GOGAT and had very low GDH to the recipient (MK 104) which lacked GLNS but had wild type levels of GOGAT and GDH. One of the transductant strains so obtained (MK 257) produced a wild type level of GLNS but had lost 2/3 of the GOGAT and almost all the GDH in a medium of excess NH_4^+. Strains deficient in GLNS (MK 93 and MK 104) produced high level of GDH even when the growth of the cells was N-limited. The GDH deficiency, (MK 270) apparently had no effect on the expression of the GLNS gene and thus produced wild type level of GLNS in excess NH_4^+. However, deficiency in GOGAT (MK 256) prevented the normal production of GLNS as the wild type did in glutamine-limitation. In view of these results, the authors concluded that the enzyme GLNS played an important role in conducting the regulation of the synthesis of enzymes responsible for glutamate formation in K. aerogenes: The GLNS itself is derepressed where ammonium or glutamine is low. The increased amount of GLNS brought about the repression of GDH which became functionally inadequate, and also relieved the catabolite repression of the enzyme histidase which was capable of forming glutamate from histidine.

The most interesting regulation of the activity of GLNS is the covalent attachment and removal of AMP from a specific tyrosyl residue in each of the enzyme's subunits. In E. coli (Stadtman et al., 1970; Ginsburg & Stadtman, 1973 and Alder et al., 1975) the ability of the GLNS to catalyze the formation of glutamine is determined by the relative state of adenylylation of the enzyme subunits. The average state of adenylylation is governed by the relative rate of adenylylation and deadenylylation catalyzed by a single enzyme adenylyltransferase (ATase), whose activity is in turn modulated by a regulatory protein P_{II}. The regulatory protein P_{II} can exist in two interconvertible forms P_{IIA} (unmodified form) and P_{IID} (uridylylated form) which respectively stimulate the adenylylation and deadenylylation activity of ATase. Conversion of P_{IIA} to P_{IID} requires UTP, α-ketoglutarate, ATP, either Mg^{++} or Mn^{++} and attachment of UMP to P_{IIA}, catalyzed by an enzyme uridylyl transferase (UTase); whereas removal of the UMP from P_{IID}, changing it back to P_{IIA}, is catalyzed by uridylyl-removing enzyme (UR-enzyme) which obligatorily requires Mn^{++}. Hence, the activity of GLNS is regulated in turn by ATase, $P_{II(A\&D)}$, UTase, UR-enzyme and various metabolites. In fact, the ultimate regulators of GLNS are certain metabolites because α-ketoglutarate and ATP not only inhibit the P_{IIA} stimulated adenylylation activity of ATase but are also required for both UTase catalyzed conversion of P_{IIA} to P_{IID} and for the P_{IID} stimulated deadenylylation activity of ATase. Glutamine and pyrophosphate, however, function in exactly the opposite way to α-ketoglutarate and ATP. It appears that a high ratio of α-ketoglutarate/glutamine, characteristic of

cells growing in N-limited medium, favours the deadenylylation of GLNS and therefore stimulate its biosynthetic activity. Conversely, a low α-ketoglutarate/glutamine ratio, characteristic of cells growing in excess of ammonium, enhances adenylylation of the enzyme, therefore rendering it inactive.

Indeed, in N_2-fixing or NH_4^+-limited K. pneumoniae and K. aerogenes (Nagatani et al., 1971; Prival et al., 1973) the enzyme GLNS is present in active, deadenylylated form whereas cells grown in excess of NH_4^+ contain mainly the inactive adenylylated GLNS.

Magasanik's group (DeLeo & Magasanik, 1975; Streicher et al., 1975 and Foor et al., 1975) studied the K. aerogenes mutants in great detail. They found that mutation sites glnA, glnB, glnD and glnE were parts of structural genes for GLNS, P_{II}, UTase and ATase respectively. Mutations in glnA4 and glnA29 resulted in production of active GLNS even in the presence of excess ammonia. Mutants having mutation in glnA51 resulted in the production of large amounts of enzymatically inactive GLNS antigen in the presence or absence of excess ammonia. Another strain carrying glnA10 mutation could not produce active enzyme or antigen at all. Moreover, cells having mutation at P_{II} and UTase (glnB and glnD mutations) failed to deadenylylate GLNS, and resulted in an abnormally low level of GLNS, as measured by antigenicity and transferase activity. On the other hand, mutation (glnE mutants) causing a

failure to adenylylate GLNS resulted in high levels of GLNS, even when the cells were grown in excess of ammonia. These results indicated that adenylylated GLNS controlled the expression of its own structural gene by repression.

Tentatively, high level of NH_4^+ favours the conversion of α-ketoglutarate to glutamine through the coupling reaction of GOGAT/GLNS, resulting adenylylation of GLNS and its own repression. Conversely, low level of NH_4^+ favours α-ketoglutarate accumulation and glutamine depletion, resulting in deadenylylation of GLNS and also derepression of GLNS. Consequently, the increasing amount of deadenylylated GLNS represses the synthesis of GDH, but activates the formation of other enzyme like histidase.

THE BASIC THEORY OF CONTINUOUS CULTURE

The theoretical aspects of continuous culture were first considered by Monod (1950), Novick & Szilard (1950) and subsequently discussed thoroughly by Herbert, Elsworth & Telling (1956). The treatment that follows is based on a recent comprehensive review by Tempest (1970).

Given the initial concentration of organism X, the specific growth rate μ and culture doubling time t_d can be described by:

$$\mu = \frac{1}{X}\frac{dX}{dt} = \frac{d(\log_e X)}{dt} = \frac{\log_e 2}{t_d} \qquad \text{Eqn 6}$$

Monod (1942) has shown that if the concentration of a single substrate S is decreased below some fixed level (a level which depends on the substrate), the growth rate of the organisms becomes dependent on the substrate concentration. Indeed the situation becomes exactly analagous to the Michaelis-Menten enzyme equation, i.e.:-

$$\mu = \mu_{max} \left(\frac{S}{K_s + S}\right) \qquad \text{Eqn 7}$$

where μ_{max} = the maximum value of μ (i.e. when S is no longer growth limiting)

K_s = saturation constant numerically equal to the substrate concentration at which $\mu = 0.5\ \mu_{max}$

The relation between growth and utilization of substrate at a constant growth rate is:-

$$\frac{dX}{dt} = -\ Y\ \frac{dS}{dt} \qquad \text{Eqn 8}$$

where Y = yield coefficient

$$= \frac{\text{wt. of bacteria formed}}{\text{wt. of substrate used}}$$

$$= -\ \frac{dX}{dS}$$

In the chemostat, the organisms are constantly being supplied with fresh medium but they are also simultaneously being washed out from the growth vessel. The net change of concentration of organism is expressed by the following balance equation:-

increase in organisms (in the growth vessel) = growth - wash out

$$\frac{dX}{dt} = \mu X - DX \qquad \text{Eqn 9}$$

where D = dilution rate

= number of complete volume change per unit time

Usually the unit time for both μ and D is 1 hour. Therefore if a complete volume change takes place in 5 hours, D is given as 0.2

From Eqn 9, it is obvious that at the situation called "steady state" (when $\frac{dX}{dt} = 0$):-

$$\mu = D = \mu_{max} \left(\frac{S}{K_s + S}\right) = \frac{\log_e 2}{t_d} \qquad \text{Eqn 10}$$

And, substituting μ from Eqn 7 in Eqn 9, we obtain:-

$$\frac{dX}{dt} = X \left(\mu_{max} \left(\frac{S}{K_s + S}\right) - D \right) \qquad \text{Eqn 11}$$

When the culture has reached a steady state at $\frac{dx}{dt} = 0$ and a steady state substrate level of $\bar{S}$, then a rearrangement of Eqn 11 gives

$$\bar{S} = K_s \left(\frac{D}{\mu_{max} - D}\right) \qquad \text{Eqn 12}$$

In the growth vessel, substrate enters at a concentration S_{in}, is partially consumed by the organisms and flows out at concentration S_{out}. The net change of substrate concentration is thus given by another balance equation:-

increase of substrate S = (input - output - consumption) (in the culture vessel)

or increase = $\left(\text{input} - \text{output} - \frac{\text{growth}}{\text{yield coef.}}\right)$

or $\frac{dS}{dt} = DS_{in} - DS_{out} - \frac{X\mu}{Y}$

or $\frac{dS}{dt} = D(S_{in} - S_{out}) - \frac{X\mu_{max}}{Y}\left(\frac{S}{K_s + S}\right)$ Eqn 13

Again at steady state, $\frac{dS}{dt} = 0$ and $\mu = D$, rearranging Eqn 13 gives:-

$$\bar{X} = Y (S_{in} - \bar{S}) \qquad \text{Eqn 14}$$

where $\bar{X}$ = concentration of organisms at the steady state

Substituting $\bar{S}$ from Eqn 12 in Eqn 14 gives:-

$$\bar{X} = Y\ (S_{in} - K_s(\frac{D}{\mu_{max} - D})) \qquad \text{Eqn 15}$$

Eqns 10, 11 and 13 define quantitatively the behaviour of a continuous culture in which the fundamental growth relations are those described by Eqn 6, 7 and 8.

Assuming the yield value Y to be independent of dilution rate D, and K_s to be small relative to S_{in}, it follows from Eqns 10, 14 and 15 that varying the dilution rate should produce changes in the steady state concentration of organisms and growth limiting substrate concentration as represented in Fig, 2.

However if K_s is large relative to S_{in}, the pattern of changes in $\bar{X}$ and $\bar{S}$ with dilution rate should be more like that in Fig. 3.

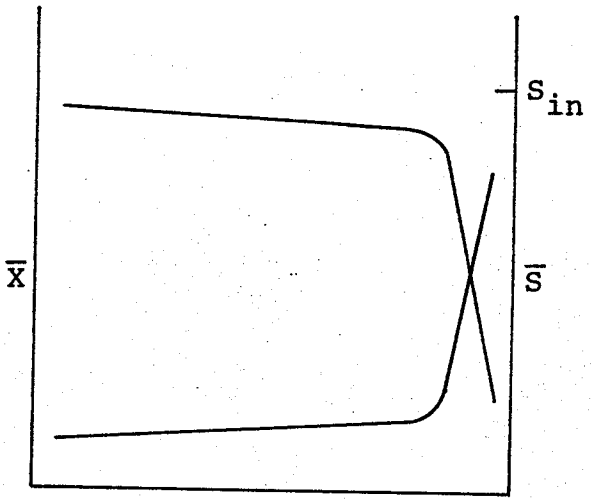


Fig. 2

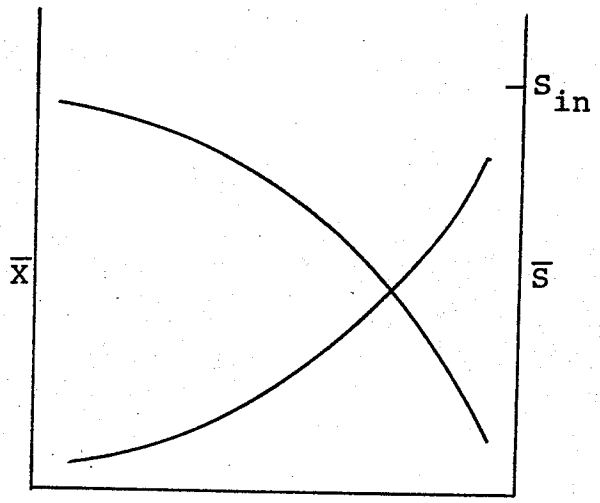


Fig. 3

Fig. 2 Influence of dilution rate on the steady-state concentration of organisms in a chemostat culture when K_s is small relative to S_r.

Fig. 3 Influence of dilution rate on the steady-state concentration of organisms in a chemostat culture when K_s is large relative to S_r.

$\bar{X}$ = concentration of organisms at the steady state

D = dilution rates $hr.^{-1}$

$\bar{S}$ = concentration of substrate at the steady state

S_{in} = input concentration of substrate

MATERIALS AND METHODS

MATERIALS AND METHODS

ORGANISM

Azotobacter chroococcum ATCC 7493 was used throughout the course of this work.

MEDIA FOR GROWTH

The N-free medium used for N_2-fixation studies was modified from Burk's medium (mannitol B_6) as described by Dalton & Postgate (1969a). When it was necessary to supplement this N-free medium with ammonium ions, ammonium in the form of either ammonium succinate or ammonium sulfate was added to give the required ammonium concentration. When ammonium salts were added to the continuous culture medium, the anion was usually metabolised more slowly than the ammonium ion was taken up (if indeed the anion was metabolised at all); consequently the pH of the culture tended to fall. With low concentrations of ammonium salts this fall could be controlled by adding extra Tris buffer to the medium, but with higher concentrations of ammonium salts, resort to automatic pH control was necessary. Therefore throughout this work the pH of the cultures was maintained at 6.6 by the dropwise addition of 1 M NaOH solution controlled (in a sterile way)

by a Radiometer combination pH electrode coupled to a Radiometer TT 11 titrator.

GROWTH AND MAINTAINANCE OF CULTURES

Lyophilized ATCC cultures were revived according to the procedure given in the instruction sheet supplied with the lyophilized culture. Purity was checked by streaking a loop-ful of growing culture onto a trypticase soy agar plate which was incubated at 28° C for a few days and then examined to make sure that only A. chroococcum colonies were present. Subsequent subculturing was done in 125 ml flasks containing about 50 ml of N-free medium and incubated at 28° C. After inoculation, the flasks were shaken on a rotary shaker at 60 oscillations per min., as pointed out by Dalton & Postgate (1969a) overaeration prevents initiation of growth. As the cultures grew thicker, they were shaken at a higher speed for one or two days before being stored in a cold room maintained at about 4° C. These cultures were then used as stock inoculation cultures for continuous culture or for subculturing.

Stock cultures were also prepared by aseptically suspending a thick growing culture in 15% glycerol solution and storing it in 2 ml portions in 2.5 ml sterile pro-vials at −60° C. When an inoculum was needed, the contents of one pro-vial were thawed and revived in the same way as above.

CONTINUOUS CULTURE APPARATUS

The continuous culture apparatus with a working volume of approximately 200 ml was based on that described by Baker (1968), Dalton & Postgate (1969a), Lees & Postgate (1973) and Hine & Lees (1976) except for the modification listed below.

The 4-litre receiver vessel was redesigned so as to replace the original cotton plug by a short length of tubing covered with metal closure inside a male ground-glass joint. This structure could be mated with a female ground-glass joint connected to the baryta percolator used for CO_2 measurement (Lees & Postgate, 1973; Hine & Lees, 1976). This modification facilitated smooth and unobstructed gas flow. A small side arm fitted with a metal closure on the neck of the flask allowed air release when the culture filled up the flask. The inside of the glass tubing between X and Y was greased before autoclaving with silicone grease to prevent liquid "creep" up the tubing. The receiver assembly is shown in Fig. 4.

STARTING A CONTINUOUS CULTURE

A flask containing about 50 ml of stock culture was used to inoculate a growth vessel containing about 150 ml N-free B_6 medium. The culture was allowed to grow overnight

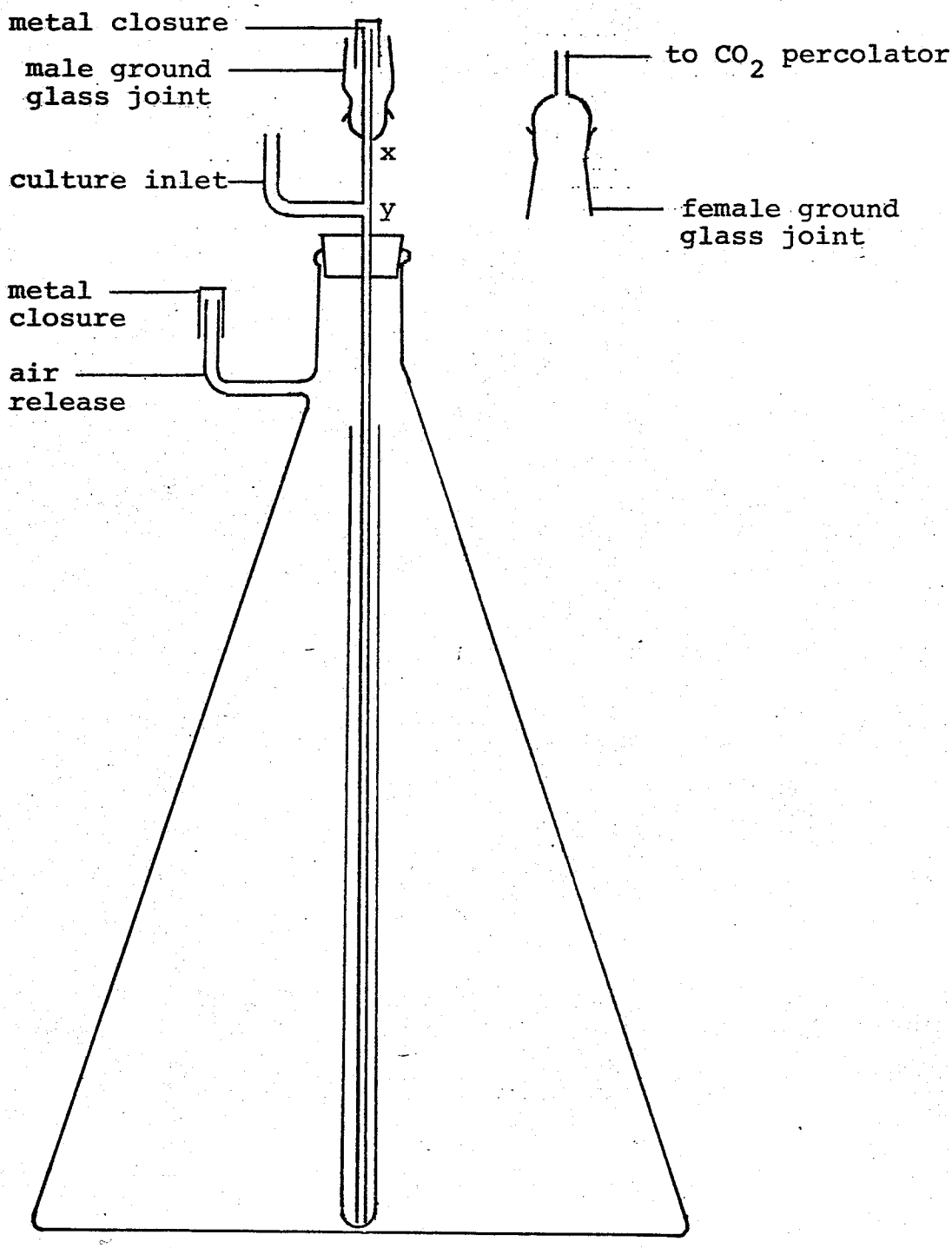


Fig. 4 The 4-litre receiver vessel.

as a batch culture with low stirring rate of 300-600 r.p.m. and an air supply of 150 ml/min.. When this culture had grown it maintained the dissolved O_2 concentration at 0 μM#, but when the stirring rate was raised to 1750 r.p.m. (vortex rate) the dissolved O_2 rapidly rose. In order to grow the bacteria at vortex stirring rate with zero dissolved O_2, a key point in this study, the air supply to the culture initially was diluted with N_2, so that the dissolved O_2 concentration did not rise to more than 10 μM. A good fast growing culture would then be able to lower the dissolved O_2 level to 0 μM in an hour or so depending on the density of the culture. Not until the O_2 concentration was lowered again to 0 μM was the N_2 supply reduced to let the dissolved O_2 come up to 10 μM again. By reducing the N_2 in the gas supply stepwise, the culture was usually able to attain and maintain in less than 24 hours the desired culture characteristics of 0 μM dissolved O_2 with 150 ml air/min. at 1750 r.p.m.. Once the culture had started to grow, fresh medium was fed in at a low dilution rate (D = 0.01 - 0.05). It was found that the dilution rate should not be increased by more than 0.05 hr^{-1} per day, otherwise the culture would very probably be washed out. Depending upon the design of the experiments, various percentages of O_2 in the gas phase could be achieved by adjusting the ratios of air, N_2 and O_2 in the gas supply. The cultures were usually allowed to reach a steady-state for at least three days.

Nominal value, limits of error 0-3 μM.

MEASUREMENT OF DISSOLVED OXYGEN CONCENTRATION IN THE CULTURE

Measurement of dissolved O_2 concentration in the culture was carried out with a steam sterilizable galvanic (Pb/Ag) type O_2 electrode connected to an oxygen meter (L. H. Engineering Co. Ltd., Bells Hill, Stoke Poges, Bucks, England). The oxygen meter in turn was connected to a Rustrak recorder (Rustrak Instrument Div., Gulton Industries, Inc., Manchester, N. H., U.S.A.) whose chart paper was set to move at 1 inch per hour. The O_2 electrode was sterilized and calibrated in a specially designed vessel containing a small amount of distilled water. The oxygen meter and the recorder were set to read zero when N_2 gas was passed into the vessel. Then the meter and recorder were set to give a scale reading of 100 in air. The full scale reading of the meter would then correspond to 20% of O_2 in the gas supply i.e. to 260 µM dissolved O_2 at 30° C, the temperature used in the experiments. The meter and recorder were then normally reset to give a full scale deflection with 5% of O_2 in the gas supply. An electrode already in use could be tested from time to time for continuing sensitivity by lifting it up aseptically above the culture surface and observing if it responded properly to exposure to the known O_2 concentration in the gas phase above the culture. Moreover the zero O_2 reading on the scale could be checked by flushing out the vessel with N_2 and observing whether the needle still read zero with the electrode above the culture. If, after extended use, the polypropylene membrane of the

electrode became coated with a bacterial film, or was broken, the sensitivity of the electrode was lost. It was then replaced by another sterile electrode. Damaged membranes on electrodes were easily replaced.

TEST FOR PURITY

The continuous culture was checked from time to time for purity by streaking a loopful of the culture in the growth vessel on a trypticase soy agar plate and incubating at 28° C for a day or two. The colonies formed were carefully examined to make sure that only A. chroococcum was present.

SAMPLING PROCEDURES AND STORAGE OF SAMPLES

Small samples about 10-12 ml were collected directly from the growth vessel into a sterile 20 ml storage bottle. The sample was preserved by adding a drop of concentrated H_2SO_4 (approx. 0.05 ml) and storing at 4° C.

Large samples, about 1 litre, were collected when required as an overflow from the growth vessel into a 1 litre Erlenmeyer flask in an insulated box filled with ice. The cells were spun down at 10,000 g for 10 min. in a Sorvall preparative centrifuge and stored in a test tube at −60° C for later use.

CHEMICALS

Highest quality chemicals from standard chemical companies were used throughout this study. Ammonium succinate was prepared by neutralizing succinic acid with ammonium hydroxide.

BIOMASS

The biomass of a culture sample was determined by centrefuging a 5 ml sample at 10,000 g for 10 min., washing once with 10 ml of glass-distilled and millipore-filtered water, recentrefuging, and then transferring the cells to a predried and preweighed aluminum dish which was subsequently placed in a drying oven at 105^{o} C for two days. The dish was cooled inside a desiccator and then reweighed. This procedure was repeated until the dry weight of the sample was constant.

CARBON DIOXIDE MEASUREMENT

The CO_2 output was measured exactly the same way as described in Lees & Postgate (1973).

MANNITOL DETERMINATION

Residual mannitol in the supernatant of the culture sample was determined by the method of periodate oxidation as described in Neish (1950).

NITROGENASE ACTIVITY ASSAY

Nitrogenase activities of the bacterial culture were determined by the acetylene reduction method developed by Hardy et al., (1968) and Postgate (1971). One ml culture, obtained directly from the growth vessel, was placed in a 34 ml volume Erlenmeyer flask sealed with a Suba-seal rubber plug. The gas phase in the flask was replaced completely by a gas mixture of 78% Ar and 22% O_2. One ml of pure acetylene gas at atmospheric pressure and temperature was injected into the flask which was then shaken at 110 strokes per min. in a 30° C water bath. After one hour of incubation, the reaction was stopped by injecting 5 drops of concentrated H_2SO_4 into the culture and the gas phase above the culture was assayed by a flame ionization gas chromatograph for any ethylene produced. Ethylene peak heights observed on the chromatogram were converted to n moles of ethylene formed at atmospheric pressure and 30° C, by comparing the peak heights from known concentrations of pure ethylene. The specific activities of the nitrogenase were expressed as n moles C_2H_4 produced/min./mg dry weight of bacteria. Standard ethylene was prepared by injecting 0.5 ml of pure ethylene at a known temperature and atmospheric pressure into an air filled 2-litre volumetric flask sealed by a Suba-seal closure.

THE FLAME IONIZATION CHROMATOGRAPHY

Ethylene was detected by a model 104 Pye vapour phase F.I.D. chromatograph, joined to a Coleman model 165 millivolt recorder. The column was a 6" X 1/8" glass tube packed with Poracil C/phenylisocyanate, thermostatted at 45° C. The flow rates of N_2 and H_2 were at 50 ml/min., while the compressed air supply to the burner was at 630 - 640 ml/min..

PREPARATION OF CELL EXTRACTS

Harvested cells were washed twice with 50 mM Tris buffer, pH 7.6 and centrifuged at 10,000 g for 10 min.. The washed cells were then made into a thick suspension with the same buffer and passed twice through the French Press at 16 K pressure. Unbroken cells and cell debris were removed by centrifugation at 10,000 g for 30 min.. More cell debris could be removed by spinning the crude extract at 100,000 g for 1 hour in a Spinco 60 Ti rotor. The top 3/4 of the clear supernatant was stored in portions at -20° C.

GLUTAMATE SYNTHASE AND GLUTAMATE DEHYDROGENASE ASSAYS

The methods were based on those described by Meers & Tempest (1970). Both glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH) were measured spectrophotometrically by recording the rate of oxidation of NADPH as a change in O.D.

at 340 nm. The reaction mixture, made up in a cuvette, contained the following:-

	1.5-2.5 mg protein (cell extract)
	0.75 μ moles NADPH
	15.00 μ moles α-ketoglutarate (Na salt)
either	15.00 μ moles L-glutamine (for GOGAT)
or	120.00 μ moles NH_4Cl (for GDH)
	Tris buffer 50 mM, pH 7.6 to final volume of 3 ml

Measurements of the decrease in O.D. at 340 nm were made with either a Shimadzu spectrophotometer or an Unicam SP700 spectrophotometer in anaerobic cuvettes of 1 cm. light path. The results were expressed in n moles NADPH oxidized/min./mg protein.

ANAEROBIC CUVETTES

Cell extracts of A. chroococcum always contained an active endogenous aerobic NADPH oxidase which interfered with the oxidation of NADPH by the enzymes GOGAT and GDH. In order to eliminate interference from this source, enzyme assays were always carried out anaerobically in the cuvette shown in Fig. 5. The substrates were placed inside the cuvette and the cell extract was placed in the bulb. The cuvette was first evacuated by a bench vacuum line and then further evacuated for 1 min. by an oil pump which could evacuate down to 10^{-4} mm Hg. The process required extreme care in shaking the cuvette rapidly at small amplitudes in order to prevent sudden boiling and

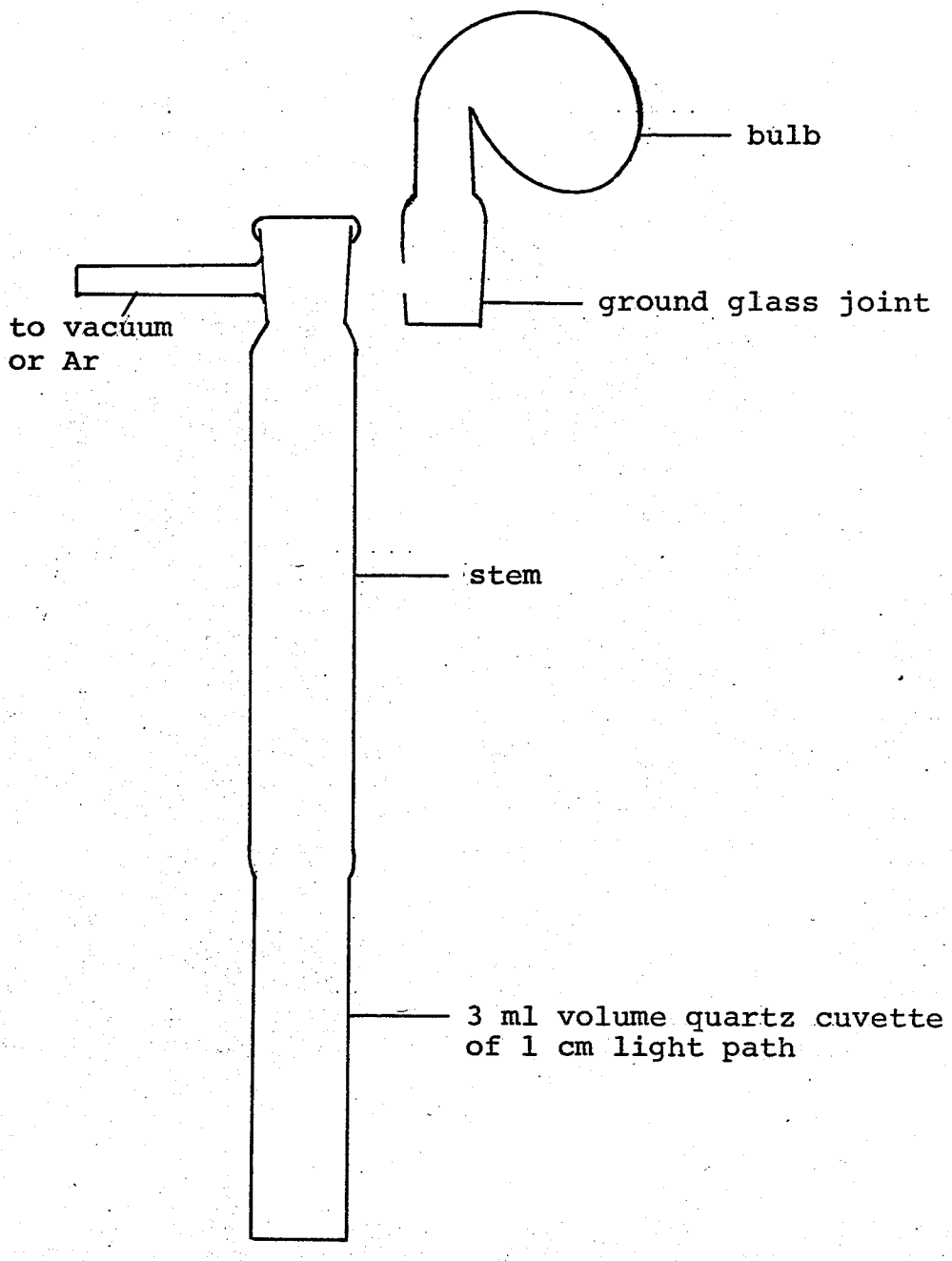


Fig. 5 The anaerobic cuvette

consequent loss of substrate or cell extract. After evacuation, the cuvette was filled with argon gas. By this time the cuvette and the materials inside had become very cold; the cuvette was therefore placed in a 27° C water bath for 15-20 sec. in order to bring the reaction temperature back to room temperature.

GLUTAMINE SYNTHETASE ASSAY

Glutamine synthetase (GLNS) is an enzyme that normally catalyses the reaction:

$$\text{L-glutamate} + NH_4^+ + \text{ATP} \longrightarrow \text{L-glutamine} + \text{ATP} + \text{Pi}$$

The enzyme can be assayed by its γ-glutamyltransferase activity

$$\text{L-glutamine} + \text{hydroxylamine} \longrightarrow \gamma\text{-glutamylhydroxamate} + \text{ammonia}$$

The enzyme exists in two forms (a) adenylylated, normally considered biologically inactive (b) deadenylylated, normally considered the biologically active form. Both forms carry out the transferase activity shown above in the presence of Mn^{++}, whereas only the deadenylylated form shows transferase activity in the presence of Mg^{++}. Therefore, measurement with Mn^{++} gives the total GLNS activity, and that with both Mn^{++} and Mg^{++} gives only the activity of the deadenylylated enzyme.

The reaction mixture contained the following:-

1.5-2.5 mg protein (cell extract)
60.00 μ moles L-glutamine
1.20 μ moles ADP
60.00 μ moles sodium arsenate
60.00 μ moles hydroxylamine-HCl
0.90 μ moles $MnCl_2$ (for both GLNS(Mn^{++}) and GLNS(Mg^{++}))
(180.00 μ moles $MgCl_2$ (for GLNS(Mg^{++}) only)
Tris buffer 20 mM, pH 7.6 to final volume of 3 ml

The "stop solution" was made up with

4.0 ml of 10% $FeCl_3$
1.0 ml of 24% trichloroacetic acid
0.5 ml 6 N HCl
and 6.5 ml water

A standard curve was prepared by using a known concentration of γ-glutamylhydroxamate to replace hydroxylamine-HCl in the above reaction mixtures. The reaction was started by adding the cell extract to the reaction mixture which was then incubated for 5 min. at 37° C. After mixing well with 0.5 ml stop solution the precipitated proteins were removed by centrifuging at 10,000 g for 10 min. and the O.D. of the clear red supernatant was measured at 540 nm in a Gilford spectrophotometer. The red colour in the supernatant was due to the complex ions formed by the Fe^{+++} and the γ-glutamylhydroxamate.

The specific activities of both GLNS(Mn^{++}) and GLNS(Mg^{++}) were expressed as μ moles of γ-glutamylhydroxamate produced/ min/mg protein.

PROTEIN DETERMINATION

The protein contents of cell extracts were estimated by the method of Lowery et al.,(1951). The O.D. of the colour developed solutions was measured at 750 nm in a Gilford spectrophotometer.

RESULTS

RESULTS

THE GROWTH OF AZOTOBACTER CHROOCOCCUM

A. chroococcum was grown in a chemostat at various dilution rates, ammonium concentrations, and under different percentages of O_2 in the gas phase, as specified. The cultures were kept at 30° C and constantly stirred at 1750 r.p.m. (vortex rate).

Actively growing cultures usually kept the dissolved O_2 concentration at zero or very close to zero. A rise of dissolved O_2 level in the culture was observed at extremely low dilution rates such as 0.05 $hr.^{-1}$ or at high dilution rates with a high percentage of O_2 in the gas phase. Cultures showing a dissolved O_2 concentration of more than 10 μM usually changed colour from milky white to pale green and then washed out. Similar changes could be observed if the pH of the culture was lowered to 4 or 5. Large amounts of lysed cells and cysts were found in cultures growing at dilution rates appreciably lower than 0.20 $hr.^{-1}$.

It proved impossible to grow a freshly inoculated continuous culture in the presence of ammonium succinate in

TABLE 1

THE EFFECT OF NH_4^+ CONCENTRATIONS IN THE MEDIA AND OF O_2 CONCENTRATIONS IN THE GAS PHASE ON THE WASH-OUT POINT OF CONTINUOUS CULTURES OF AZOTOBACTER CHROOCOCCUM

NH_4^+ conc.	O_2 conc. (%)		
	D = 0.22	D = 0.30	D = 0.35
N_2-fixing	>50%[a]	50%[a]	W.O.[b]
1 mM	30%	W.O.	W.O.
4 mM	N.D.[c]	N.D.	30%
20 mM	>20%	W.O.	W.O.
30 mM	>10%	W.O.	W.O.
50 mM	<5%	W.O.	W.O.

a from Hine, 1975.
b wash-out
c not determined

B_6 medium because as soon as the stirring rate was increased to 1750 r.p.m. the pH of the culture dropped to 4 or 5 thus preventing further growth. As a consequence, automatic pH control was necessary. When high concentrations of ammonium were used it was very difficult to follow the growth rate of the organisms by increasing the dilution rates. At low dilution rates, the cultures were unstable, and the large amounts of lysed cells and cysts made biomass determinations almost

meaningless, while higher dilution rates, or higher O_2 levels, usually led to wash out. As Table 1 shows, the nitrogen fixing cultures stood up to 50% O_2 at a dilution rate of 0.22 $hr.^{-1}$ while the culture fed with 50 mM NH_4^+ medium at the same dilution rate could not tolerate an O_2 level even as low as 5%.

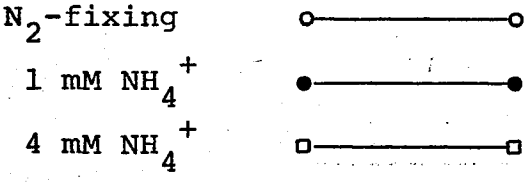
VARIATION OF BIOMASS WITH CHANGING GROWTH CONDITIONS

The variations of biomass with changes of dilution rates and O_2 concentrations and with different concentrations of NH_4^+ are shown in Fig. 6 and 7.

Fig. 6 shows that the biomass of N_2-fixing cultures decreased very slightly with increase in dilution rate, whereas cultures containing 1 mM and 4 mM NH_4^+ showed a biomass maximum at D = 0.22. When D was further increased the biomass came down to levels similar to those of the N_2-fixing cultures. The change of biomass with dilution rate of cultures grown in 20 and 30 mM NH_4^+ was not followed because of the difficulties mentioned in the last section.

In Fig. 7, the change in biomass at D = 0.22 is related to the O_2 concentration in the gas supply and the ammonium concentration in the medium. It will be seemed in general an increase of O_2 concentration or an increase of NH_4^+ concentration results in a higher growth rate of the organisms;

Fig. 6 Variation in biomass with different dilution rates of continuous cultures of A. chroococcum and with different concentrations of NH_4^+ at 20% O_2.



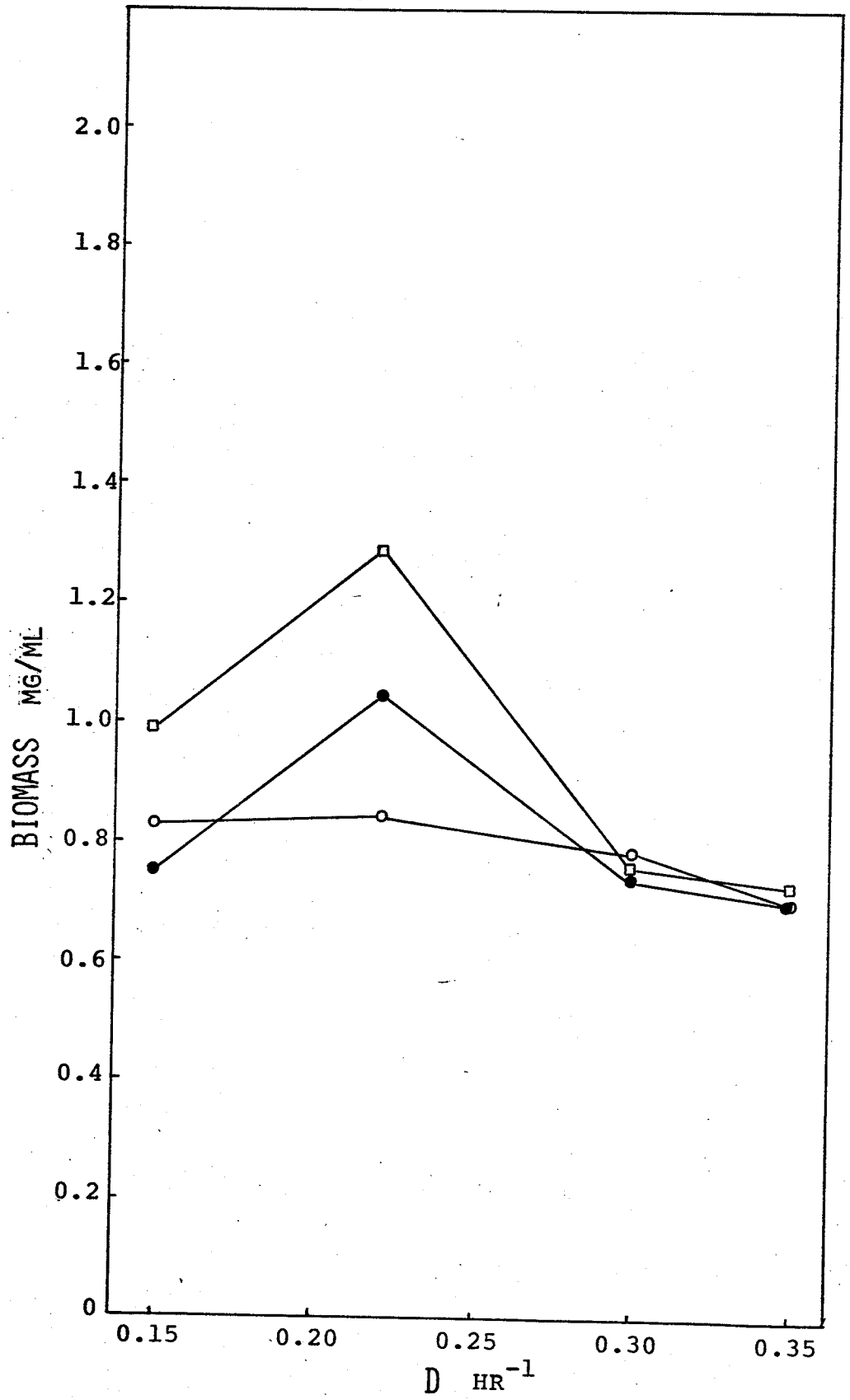
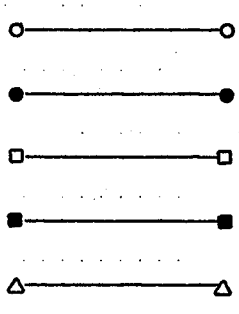
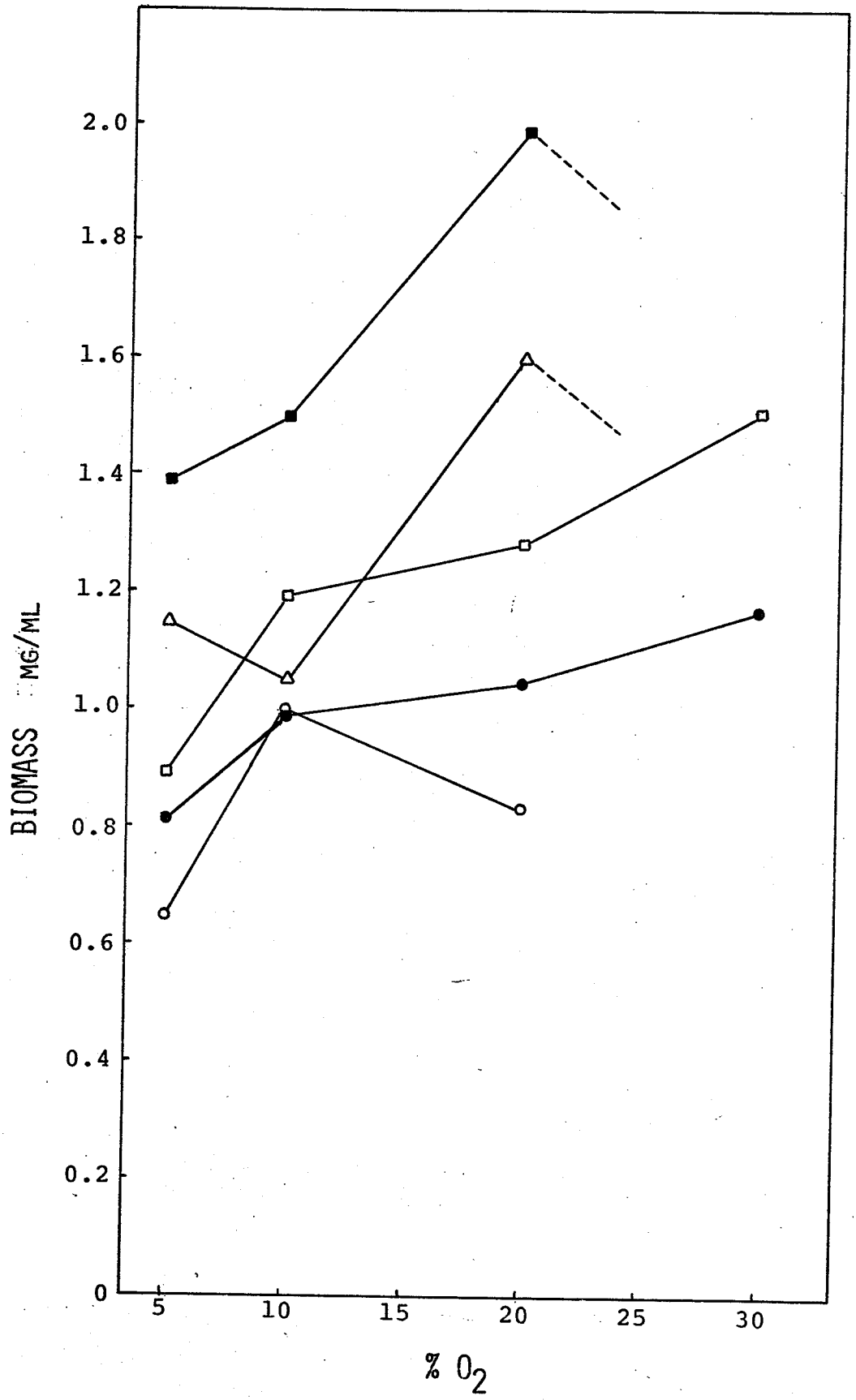

BIOMASS MG/ML
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.15
0.20
0.25
0.30
0.35
D HR-1

Fig. 7 Variation in biomass with different concentrations of O_2 of continuous cultures of A. chroococcum and with different concentrations of NH_4^+ at D = 0.22.



Dashed lines represent transitions to "unsteady" or "wash-out" state.


BIOMASS MG/ML
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
% O_2

the fall-off in growth (wash-out) shown by the 20 and 30 mM NH_4^+ cultures at O_2 concentration greater than 20% illustrates the difficulties mentioned in the last section.

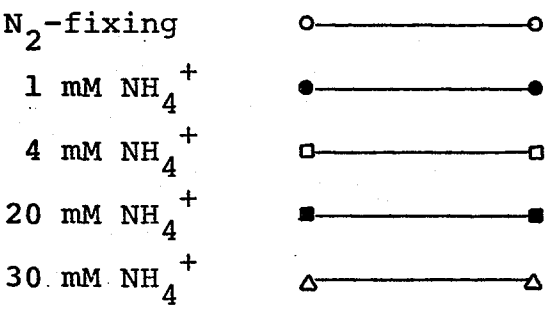
VARIATION OF RESIDUAL MANNITOL WITH CHANGING GROWTH CONDITIONS

Fig. 8 illustrates the variation of residual mannitol (initial conc. = 10 mg/ml) with the percentage of O_2 in the gas phase at D = 0.22 and also with different concentrations of NH_4^+. The amounts of residual mannitol decreased more or less linearly with any increase of O_2%. However, none of the cultures used more than 9 mg of mannitol/ml. The residual mannitol also increased with any increase in the dilution rate irrespective of other growth conditions (not shown).

VARIATION OF CO_2 PRODUCTION WITH CHANGING GROWTH CONDITIONS

The pattern of change of CO_2 output per litre culture per hour with changes in D at 20% O_2 is shown in Fig. 9. The CO_2 output per litre culture per hour under N_2-fixing conditions, was steady from D = 0.22 to D = 0.35. With 1 and 4 mM NH_4^+, the CO_2 outputs were only slightly greater. Fig. 10 depicts the effect of O_2 concentrations on CO_2 output at D = 0.22. Invariably, the CO_2 outputs increased with the rise of O_2 concentrations in the gas phase. The slight decrease of CO_2 production at 10% O_2 from cultures grown in 20 and 30 mM NH_4^+ is probably due to fluctuations of the pressure in the air supply.

Fig. 8 Variation in residual mannitol concentration with different concentrations of O_2 of continuous cultures of A. chroococcum and with different concentrations of NH_4^+ at D = 0.22.



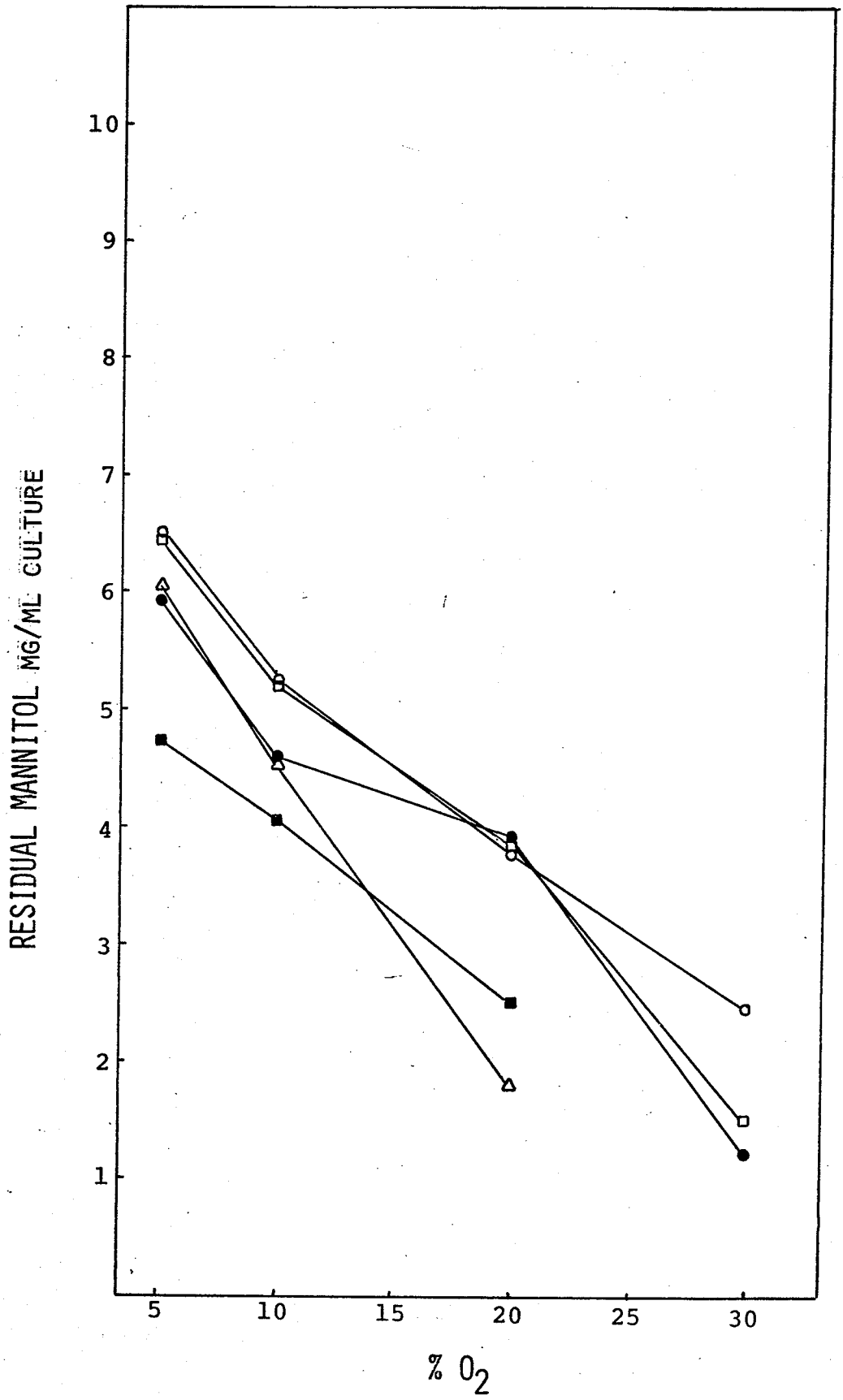
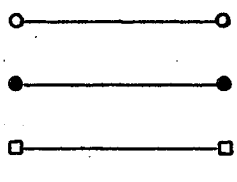

RESIDUAL MANNITOL MG/ML CULTURE
10
9
8
7
6
5
4
3
2
1
5
10
15
20
25
30
% O_2

Fig. 9 Variation in CO_2 output per 1. culture per hr. with different dilution rates of continuous cultures of A. chroococcum and with different concentrations of NH_4^+ at 20% O_2.



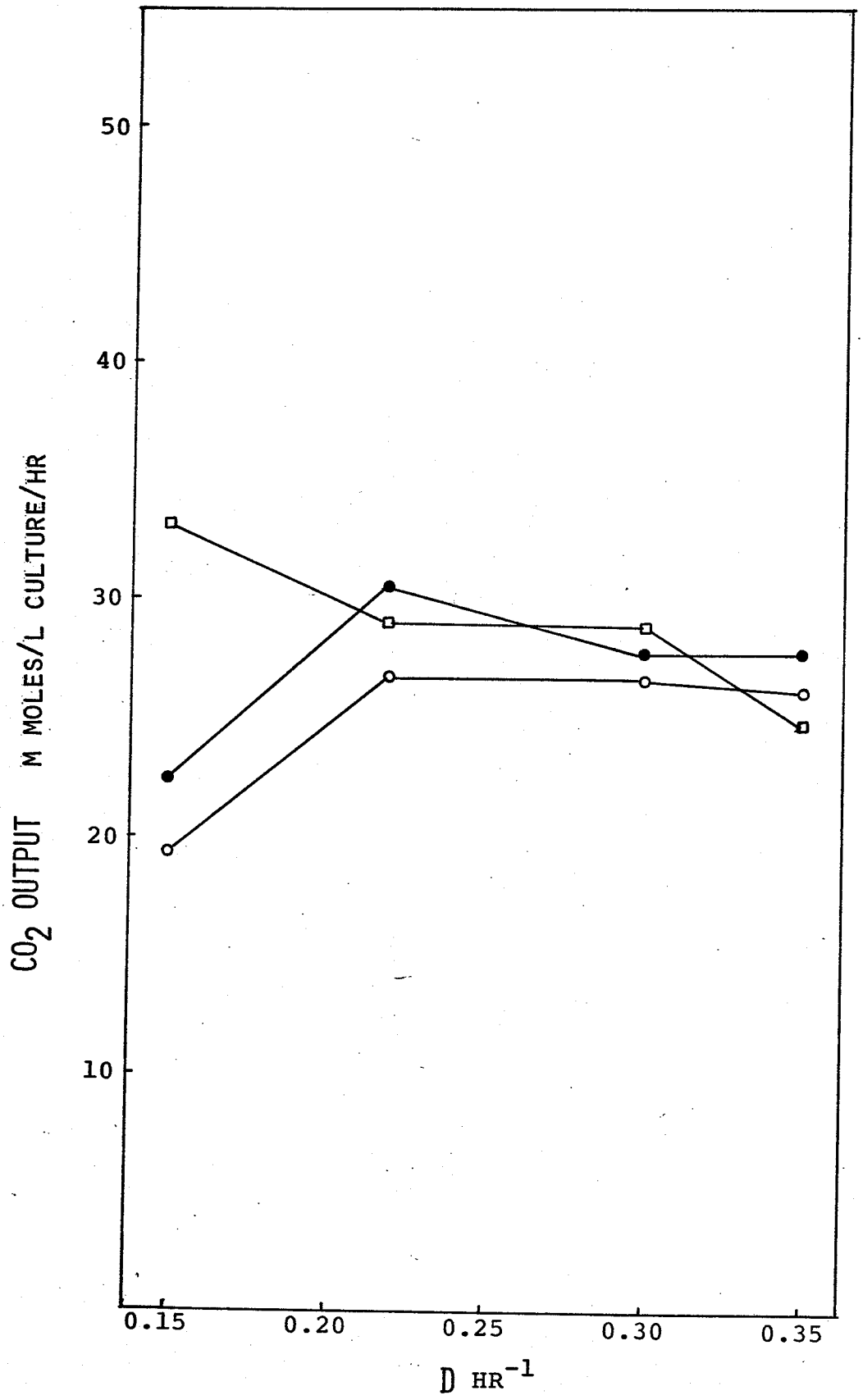
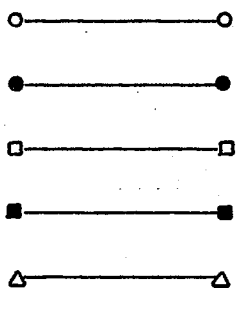
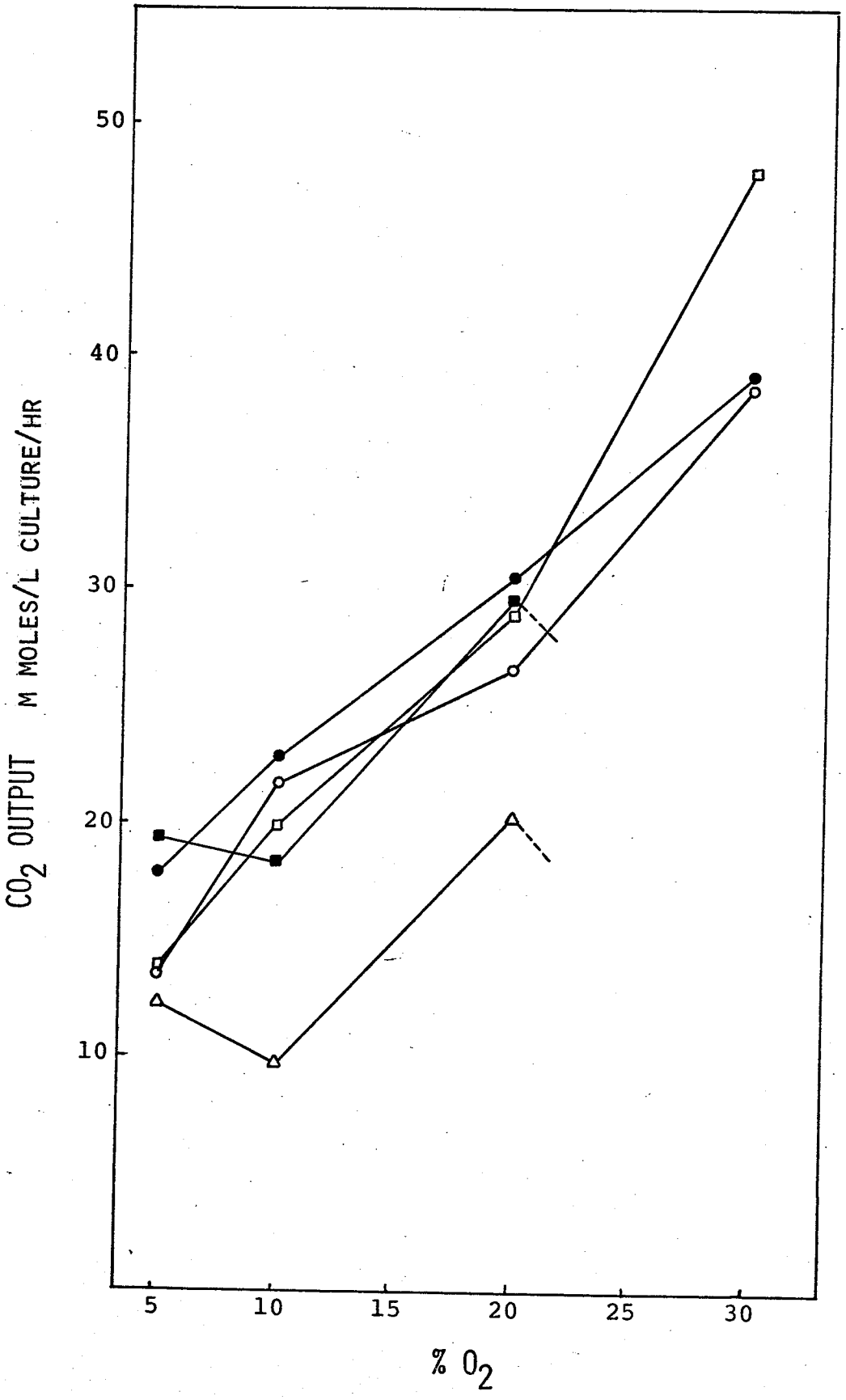

50
40
30
20
10
CO_2 OUTPUT M MOLES/L CULTURE/HR
0.15
0.20
0.25
0.30
0.35
D HR^{-1}

Fig. 10 Variation in CO_2 output per l. culture per hr. with different concentrations of O_2 of continuous cultures of A. chroococcum and with different concentrations of NH_4^+ at D = o.22.



Dashed lines represent transitions to "unsteady" or "wash-out" states.


50
40
30
20
10
CO_2 OUTPUT M MOLES/L CULTURE/HR
5
10
15
20
25
30
% O_2

A better way to express the rates of CO_2 production is to divide the CO_2 outputs (mM CO_2/l. culture/hr.) by cell growth (mg dry wt./l. culture x dilution rate in $hr.^{-1}$). This gives a dimensionless number called the Respiratory Index (R.I.) (Hine & Lees, 1976) representing the m moles CO_2 produced per milligram cell growth. The variations of R.I. with O_2 concentration from cultures grown at D = 0.22 with various NH_4^+ concentrations are shown in Fig. 11. At 20% O_2 the R.I. decreased with increasing concentrations of NH_4^+, however, at 5% and 10% O_2 the order was reversed with the cultures fixing N_2 and in 1 mM NH_4^+. Another important point that this Fig. shows was that cultures fixing N_2 or growing in low NH_4^+ were able to change their R.I. rapidly with change of O_2 concentration, while those growing in high NH_4^+ concentrations were not.

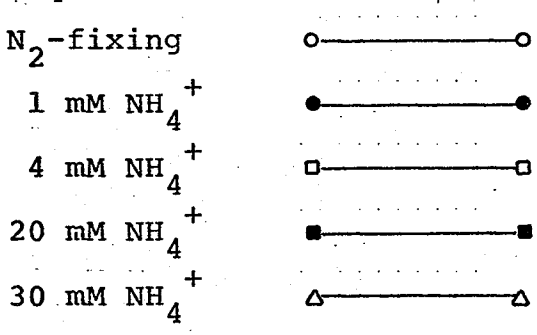
VARIATIONS OF TOTAL CARBON BALANCE

The total carbon input to the cultures is assumed to be balanced by the corresponding total carbon output according to the following equation; an assumption not precisely (valid unless all oxidized mannitol is converted to CO_2 :-)

$$C_{mannitol} = C_{residual\ mannitol} + C_{CO_2} + C_{cell} \qquad \text{Eqn 16}$$

The calculated carbon inputs and outputs at different growth conditions are plotted in Fig. 12. The horizontal line represents the carbon input at D = 0.22.

Fig. 11 Variation in the Respiratory Index with different concentrations of O_2 of continuous cultures of A. chroococcum and with different concentrations of NH_4^+ at D = 0.22.



Dashed lines represent transitions to "unsteady" or "wash-out" states.

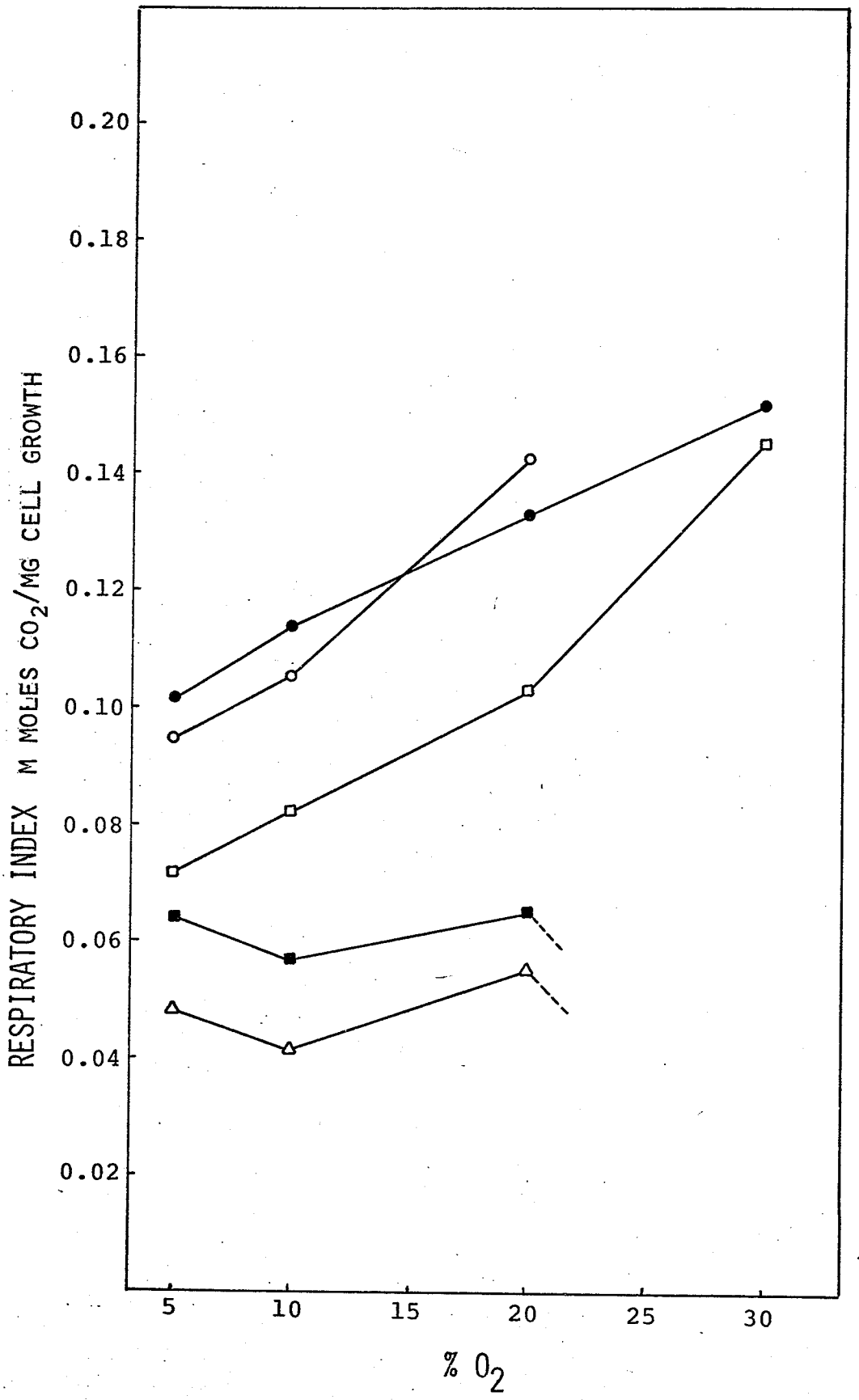
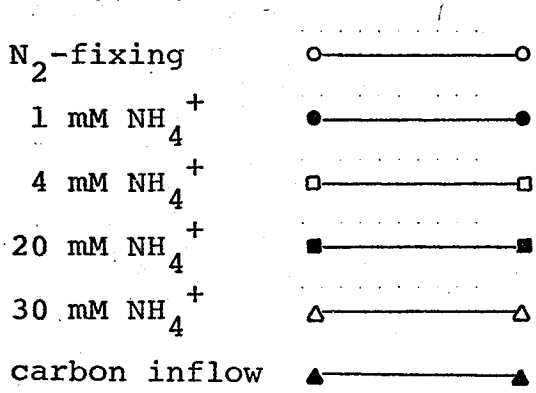
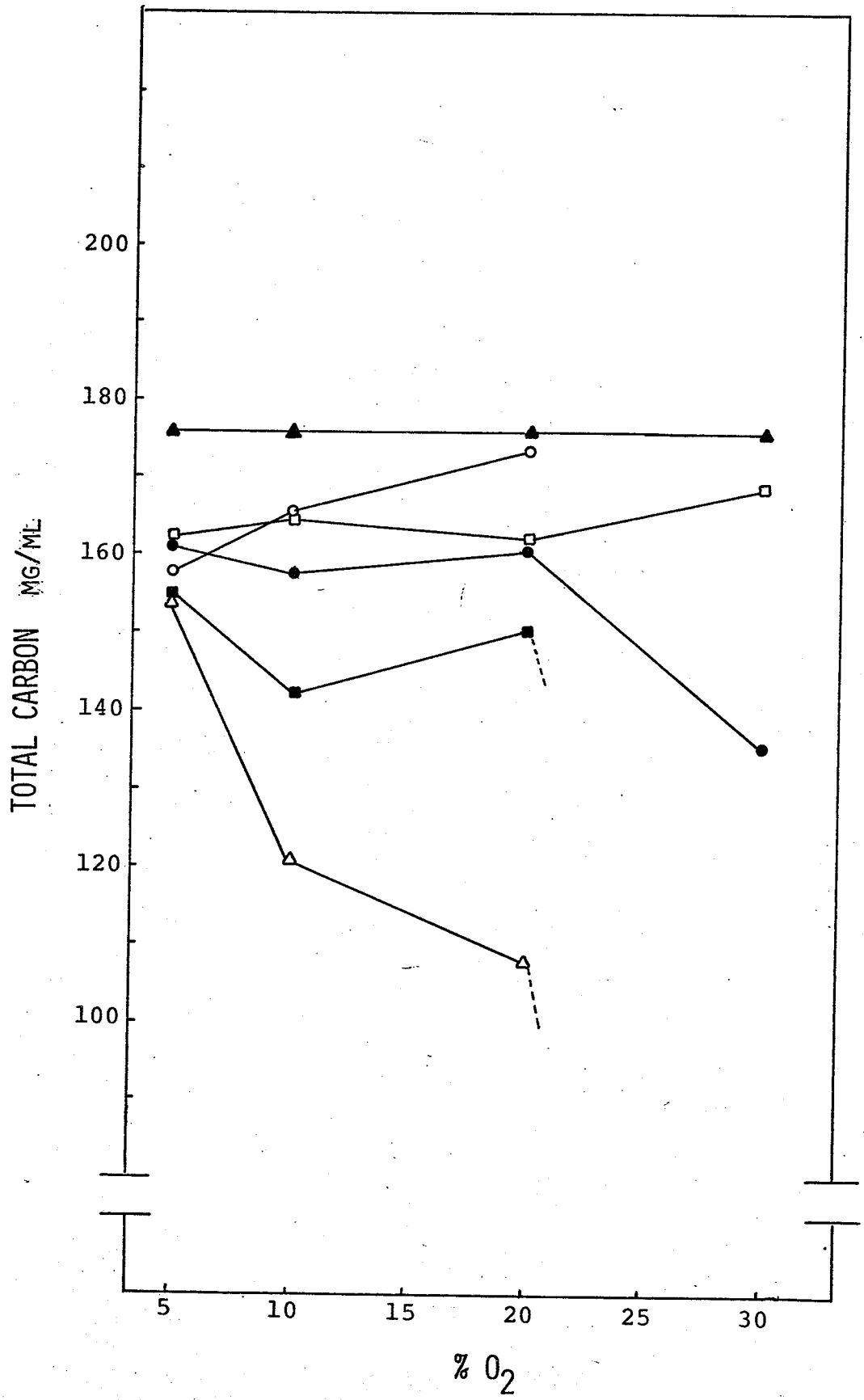

RESPIRATORY INDEX M MOLES CO_2/MG CELL GROWTH
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
5
10
15
20
25
30
% O_2

Fig. 12 Variation of total carbon balance with different concentrations of O_2 of continuous cultures of A. chroococcum and with different concentrations of NH_4^+ at D = 0.22.



Dashed lines represent transitions to "unsteady" or "wash-out" states.


TOTAL CARBON MG/ML
200
180
160
140
120
100
5
10
15
20
25
30
% O_2

Only the carbon output of the N_2-fixing culture at 20% O_2 comes very close to the carbon input. There was no significant difference between results from cultures fixing N_2 and those growing in low concentrations of NH_4^+. The deviation of the value from 1 mM NH_4^+ at 30% O_2 was probably because of the instability of the culture at wash-out point. However, the carbon outputs from cultures grown in 20 and 30 mM NH_4^+ and above 10% O_2 were significantly lower than the input on the basis of the balance equations given above.

VARIATION OF NITROGENASE, GOGAT, GLNS AND GDH WITH DILUTION RATES

Figs. 13,14 and 15 show the variations of these enzymes in cells grown at different dilution rates at 20% O_2 in media containing zero, 1 and 4 mM NH_4^+.

(a) N_2-fixing conditions

In N_2-fixing cultures, as Fig. 13 shows, the nitrogenase increased by 80% when D was increased from 0.22 to 0.35, while the NH_4^+ assimilating enzymes (GOGAT, GLNS and GDH) remained fairly constant. The GOGAT level observed is very low compared with that in A. vinelandii (Nagatani et al., 1971), but about the same as that in another strain of A. chroococcum (Drozd et al., 1972). The amount of GDH which was comparable with that of GOGAT in the A. chroococcum strain studied by

Drozd et al. (1972) was barely detectable in the strain used in the present work. Magnesium ions stimulated the activity of the deadenylylated GLNS in much the same way as it did in K. aerogenes (Prival et al., 1973). Therefore, the GLNS (Mg^{++}) activity should not be regarded as the absolute level of the enzyme, rather the relative level of deadenylylated portion of the GLNS in the organism.

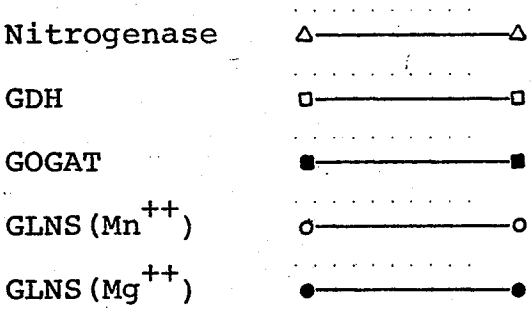
(b) 1 mM NH_4^+

The pattern of the change of these enzymes in cultures growing in 1 mM NH_4^+, as shown in Fig. 14, was not markedly different from that in N_2-fixing cultures (Fig. 13). The inhibitory effect of the NH_4^+ on the nitrogenase (Burris, 1971) was not seen here and moreover, the enzyme increased by 6 fold from D = 0.22 to 0.35, eventually reaching about the same level as was found in N_2-fixing cultures.

(c) 4 mM NH_4^+

Fig. 15 illustrates the somewhat different pattern of changes that occurred in the presence of 4 mM NH_4^+. The nitrogenase, GOGAT and GLNS reached peak levels at D = 0.30, while the GDH, which was fairly active, remained reasonably constant throughout the range of dilution rates tested. From D = 0.30 to 0.35, there was apparently a change in the relative amounts of the adenylylated and deadenylylated GLNS, as shown by the crossover of the lines.

Fig. 13 Variation of enzymes activities with different dilution rates of continuous cultures of A. chroococcum under N_2-fixing condition and at 20% O_2.



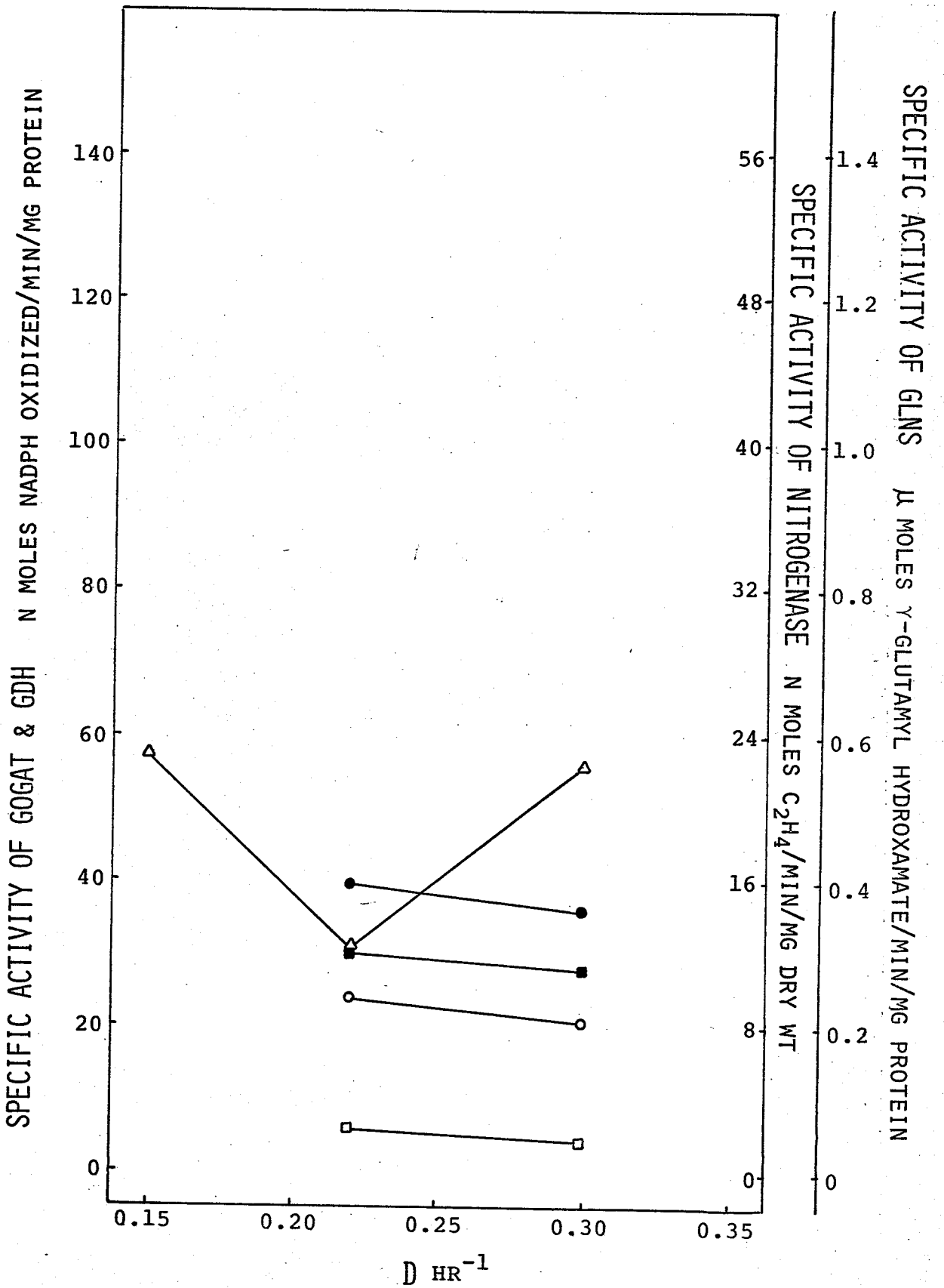
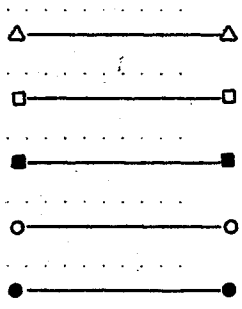

SPECIFIC ACTIVITY OF GOGAT & GDH N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
D HR^{-1}
0
20
40
60
80
100
120
140
0
8
16
24
32
40
48
56
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0.15
0.20
0.25
0.30
0.35

Fig. 14 Variation of enzymes activities with different dilution rates of continuous cultures of A. chroococcum at 1 mM NH_4^+ and at 20% O_2.



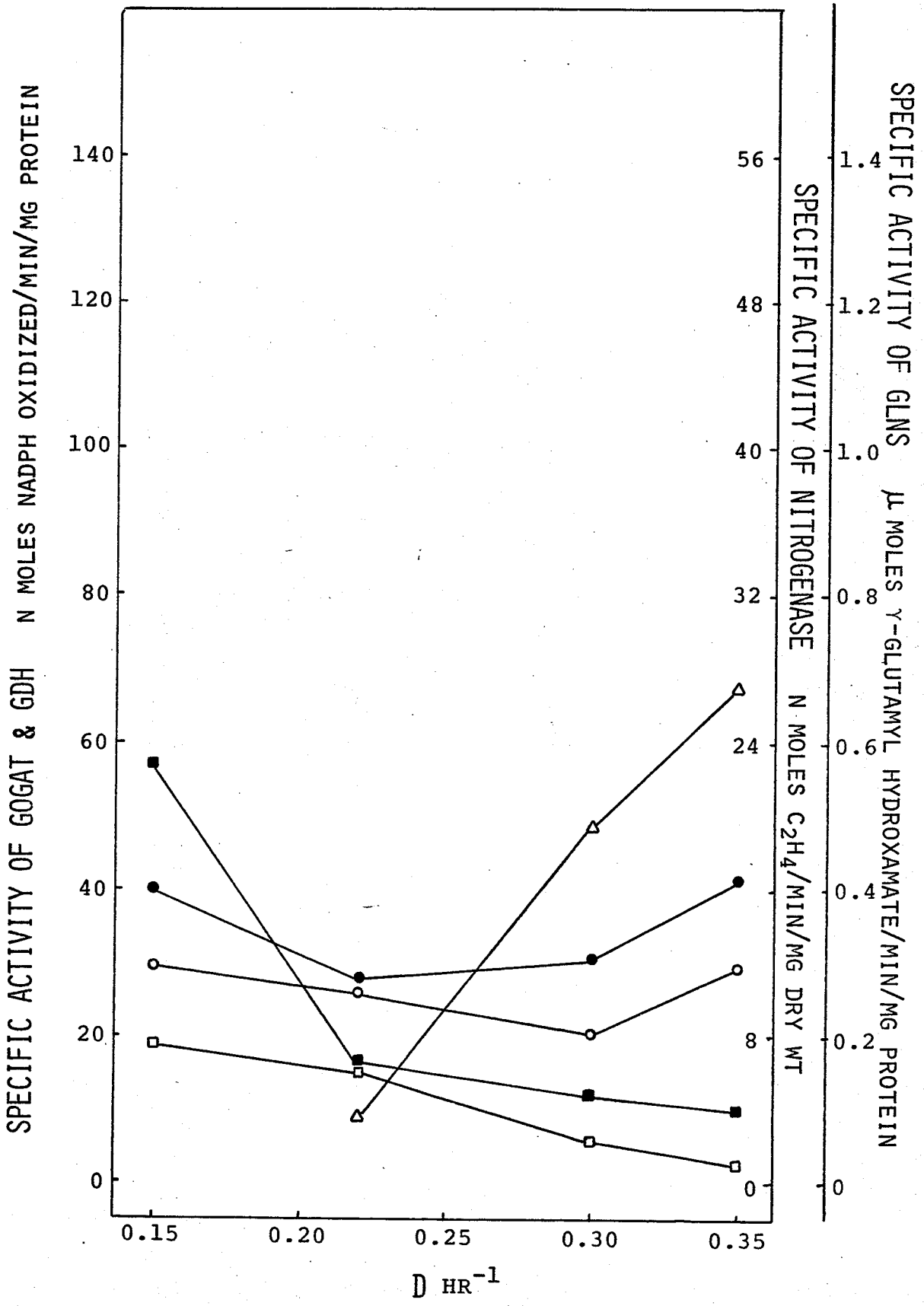
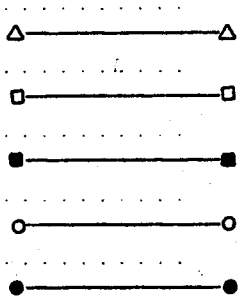
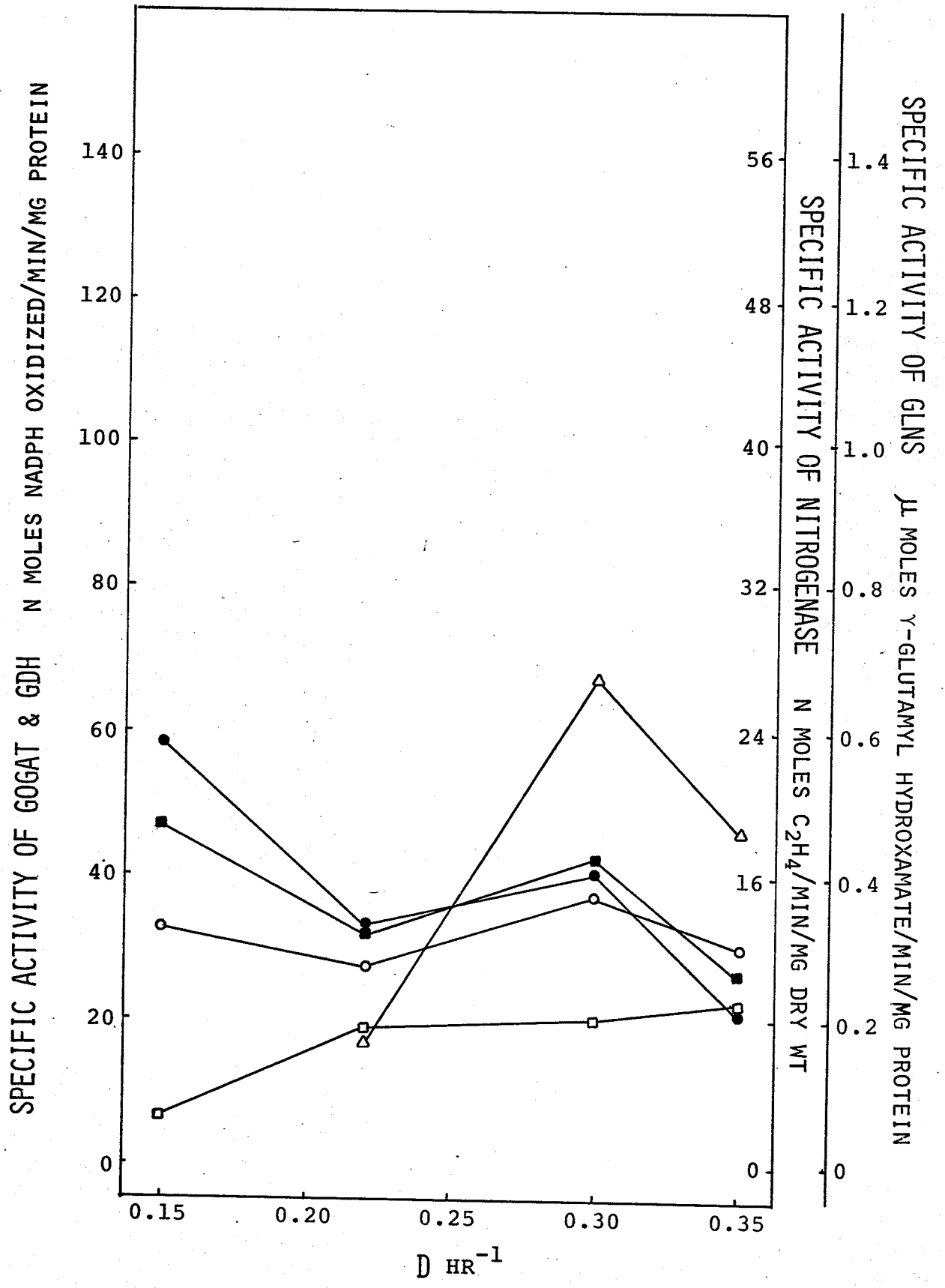

SPECIFIC ACTIVITY OF GOGAT & GDH N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
D HR^{-1}
140
120
100
80
60
40
20
0
56
48
40
32
24
8
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0.15
0.20
0.25
0.30
0.35

Fig. 15 Variation of enzymes activities with different dilution rates of continuous cultures of A. chroococcum at 4 mM NH_4^+ and at 20% O_2.




SPECIFIC ACTIVITY OF GOGAT & GDH N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μL MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
D HR^{-1}
0
20
40
60
80
100
120
140
0
16
24
32
40
48
56
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0.15
0.20
0.25
0.30
0.35

VARIATION OF NITROGENASE, GOGAT, GLNS AND GDH WITH CHANGES IN THE O_2 CONCENTRATION

(a) N_2-fixing conditions

Under N_2-fixing conditions (Fig. 16) the nitrogenase activity of the organism steadily declined as the O_2 concentration was increased. The GOGAT levels followed the similar pattern. Between 5 and 10% O_2, there was a changeover between the two forms of GLNS. The amount of the deadenylylated form of the GLNS rose, the amount of the adenylylated GLNS fell, while the total amount of GLNS remained reasonably constant. The GDH level was low throughout.

(b) 1 mM NH_4^+

The variation in these same enzymes when the medium is supplemented with 1 mM NH_4^+ is shown in Fig. 17. The overall pattern was quite similar to that shown under N_2-fixation conditions.

(c) 4 mM NH_4^+

In the presence of 4 mM NH_4^+, the enzymes variation however show a rather different pattern (Fig. 18). The nitrogenase fluctuated and reached the highest level at 30% O_2. The GOGAT stayed fairly constant at the same level as

it was under N_2-fixing condition with 20 and 30% O_2 (c.f. Fig. 16). The GDH appeared at a very high level initially, but decreased to a low level as the O_2 concentration was increased. The total amount of GLNS remained fairly constant but the deadenylylated portion fluctuated.

(d) 20 mM NH_4^+

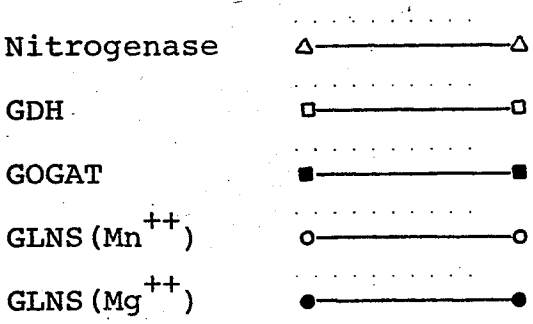
As Fig. 19 shows the situation when the culture was grown in 20 mM NH_4^+ is markedly different. Both the nitrogenase and GOGAT were present in low but significant amounts. The GDH which was very high initially had lost almost 2/3 of its activity when the O_2 was increased from 5 to 20%. The level of GLNS dropped by 50% when the O_2 concentration was raised from 5% to 20%, and the relative amount of the deadenylylated enzyme was low throughout.

(e) 30 mM NH_4^+

As Fig. 20 shows, under 30 mM NH_4^+, the overall enzyme activities have a pattern that seemed to be quite bizarre The nitrogenase had almost disappeared, the GOGAT had risen markedly and the GDH attained levels 1 order higher than had been noted at lower concentration of NH_4^+. O_2 toxicity, (see The Effect of Oxygen)# prevented investigation at higher O_2 levels.

see page 11

Fig. 16 Variation of enzymes activities with different concentrations of O_2 of continuous cultures of A. chroococcum under N_2-fixing condition and at D = 0.22.



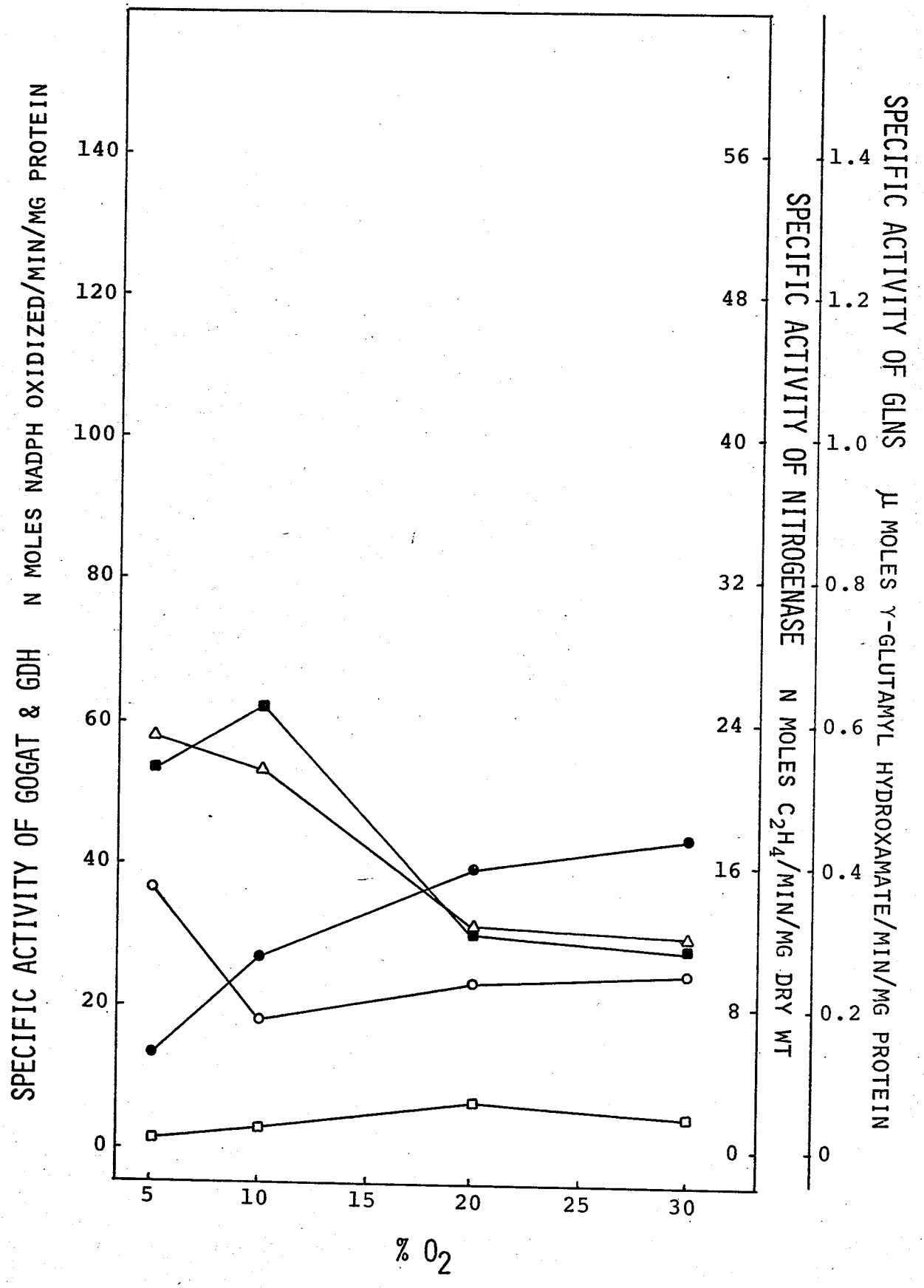
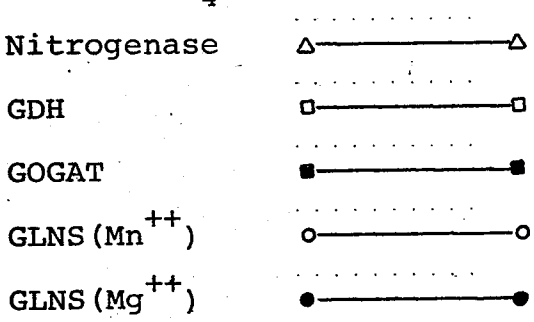

SPECIFIC ACTIVITY OF GOGAT & GDH N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
% O_2
0
20
40
60
80
100
120
140
8
16
24
32
40
48
56
0.2
0.4
0.6
0.8
1.0
1.2
1.4
5
10
15
20
25
30

Fig. 17 Variation of enzymes activities with different concentrations of O_2 of continuous cultures of A. chroococcum at 1 mM NH_4^+ and at D = 0.22.



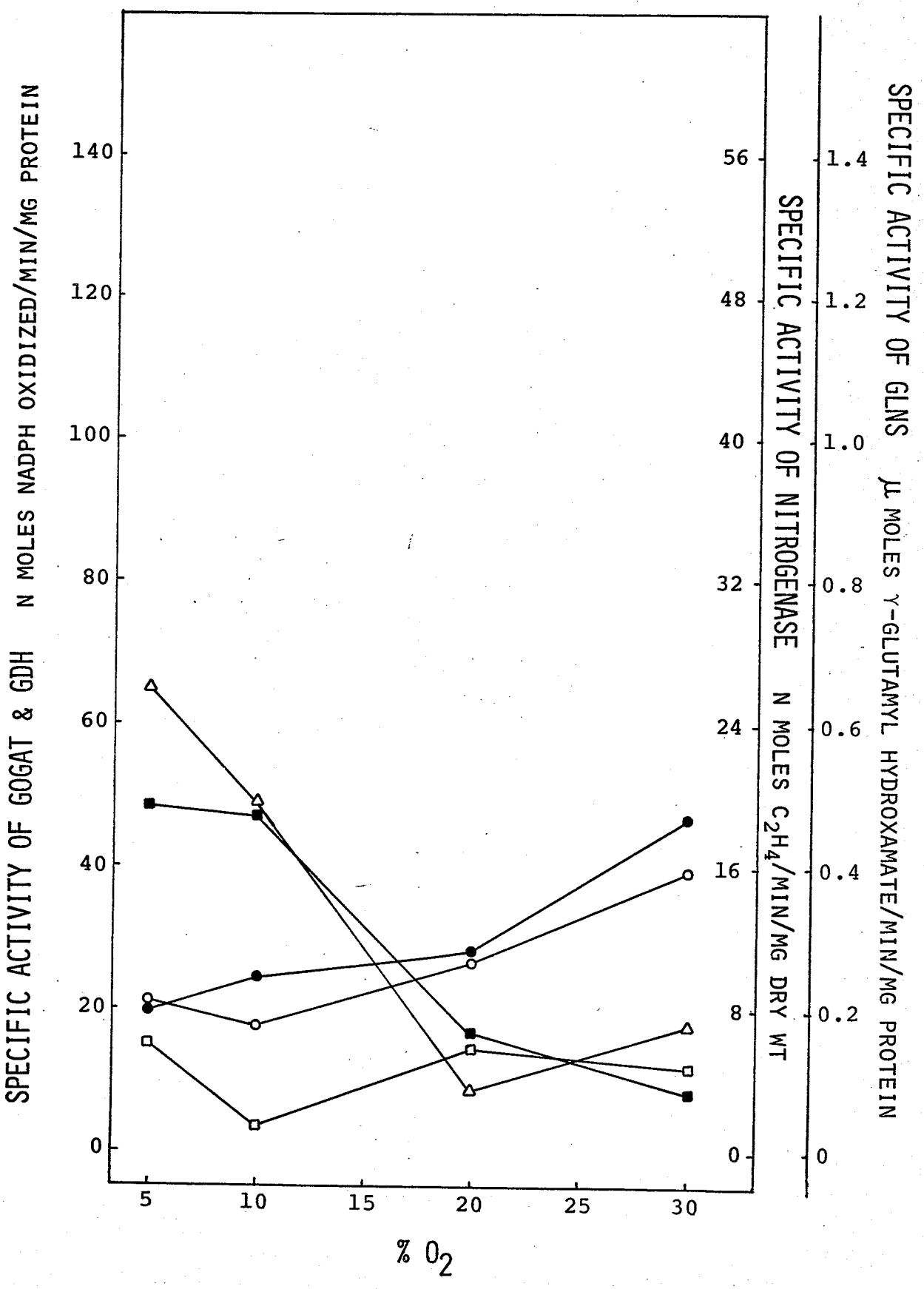
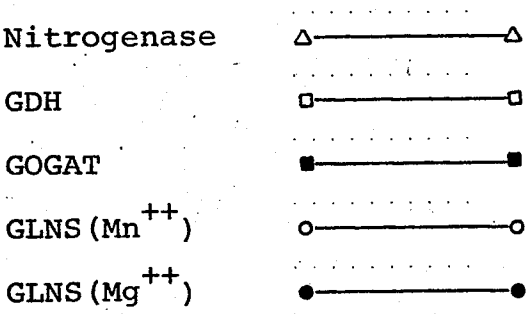

SPECIFIC ACTIVITY OF GOGAT & GDH N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
140
120
100
80
60
40
20
0
56
48
40
32
24
16
8
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
% O_2

Fig. 18 Variation of enzymes activities with different concentrations of O_2 of continuous cultures of A. chroococcum at 4 mM NH_4^+ and at D = 0.22.



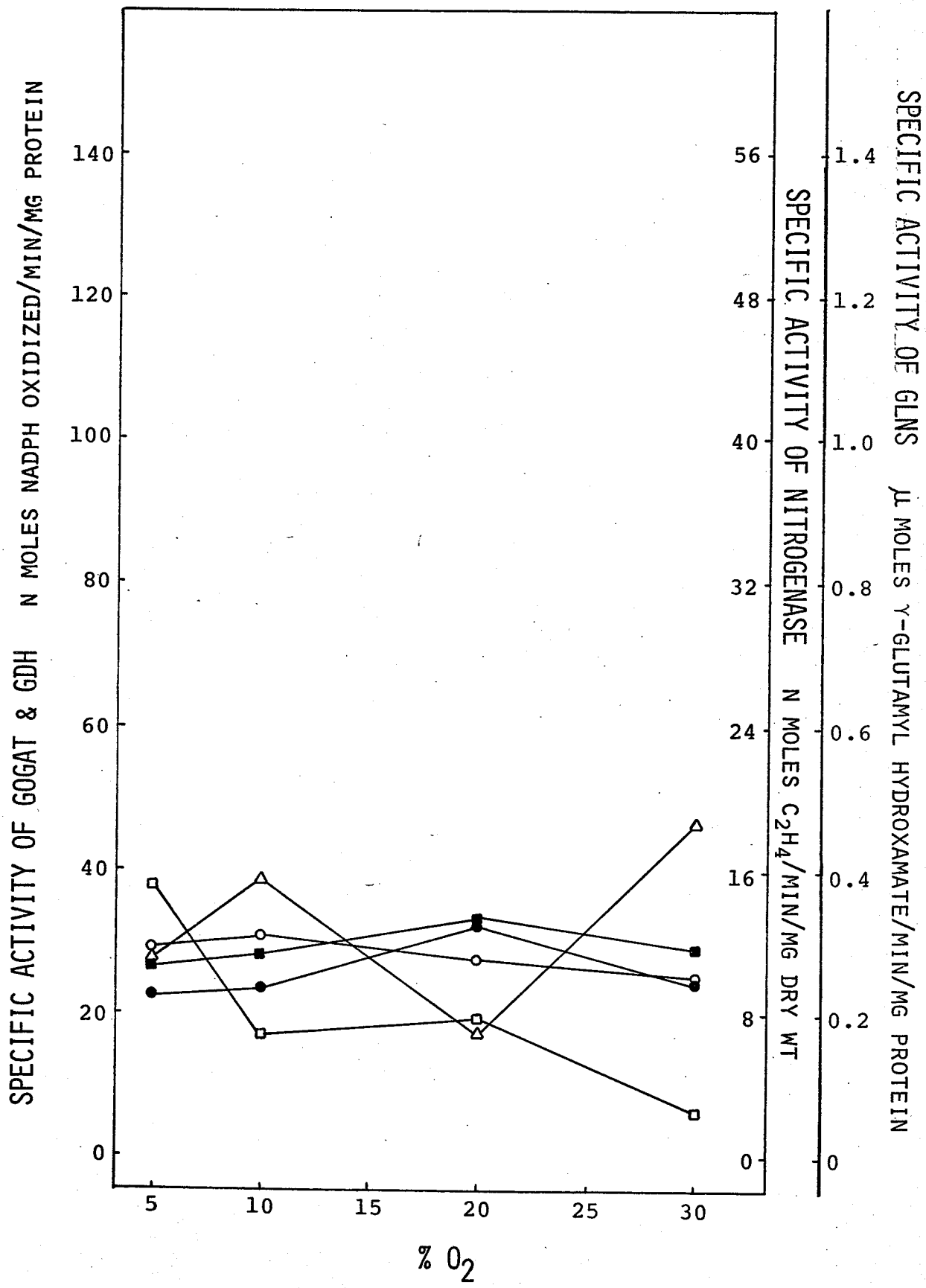
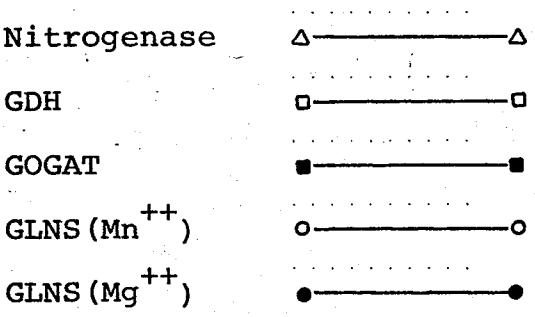

SPECIFIC ACTIVITY OF GOGAT & GDH N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
% O_2
0
20
40
60
80
100
120
140
8
16
24
32
40
48
56
0.2
0.4
0.6
0.8
1.0
1.2
1.4
5
10
15
20
25
30

Fig. 19 Variation of enzymes activities with different concentrations of O_2 of continuous cultures of A. chroococcum at 20 mM NH_4^+ and at D = 0.22.



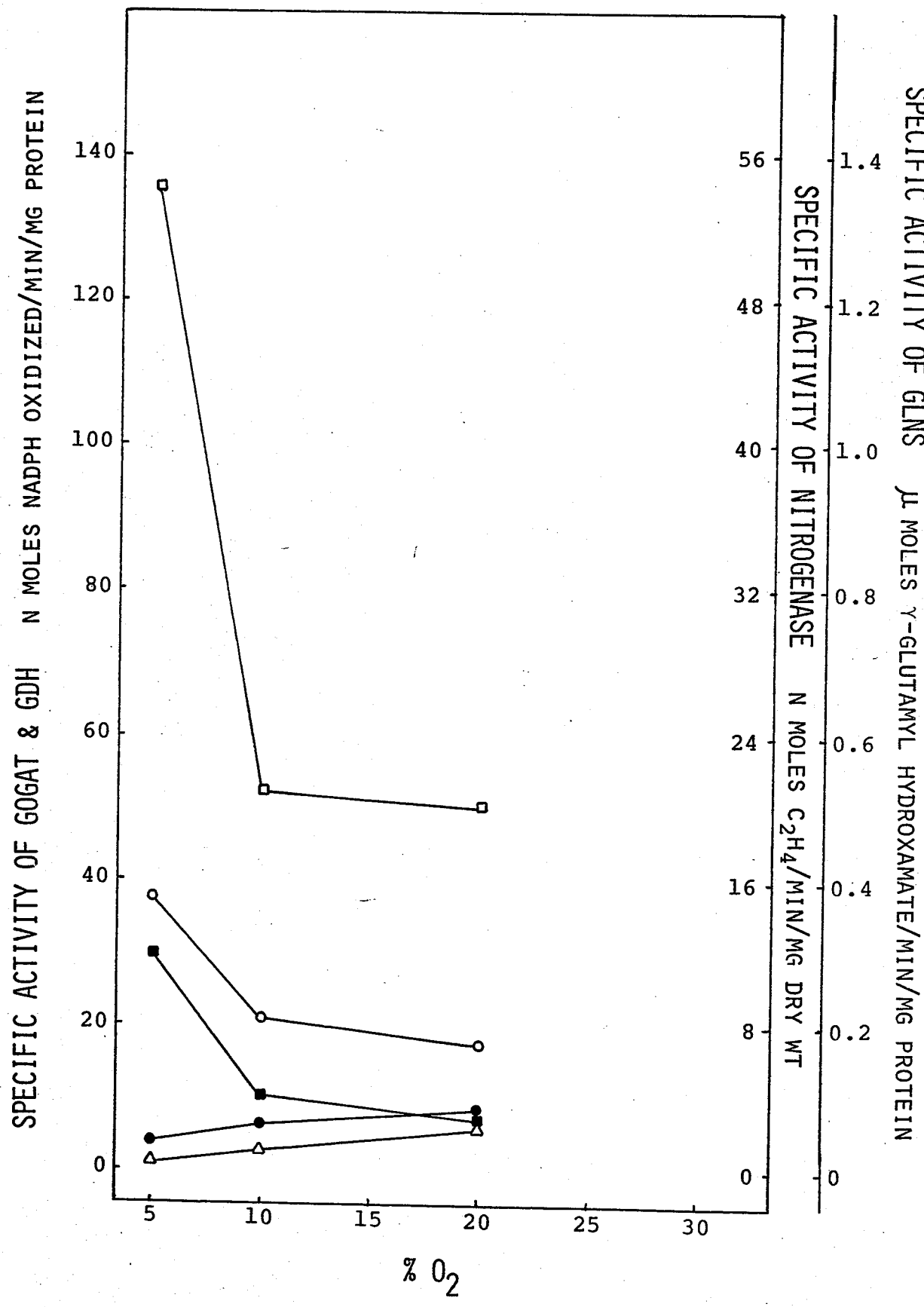
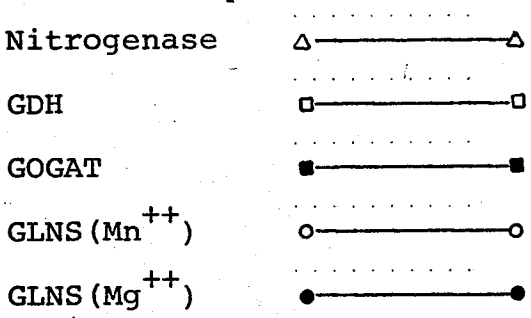
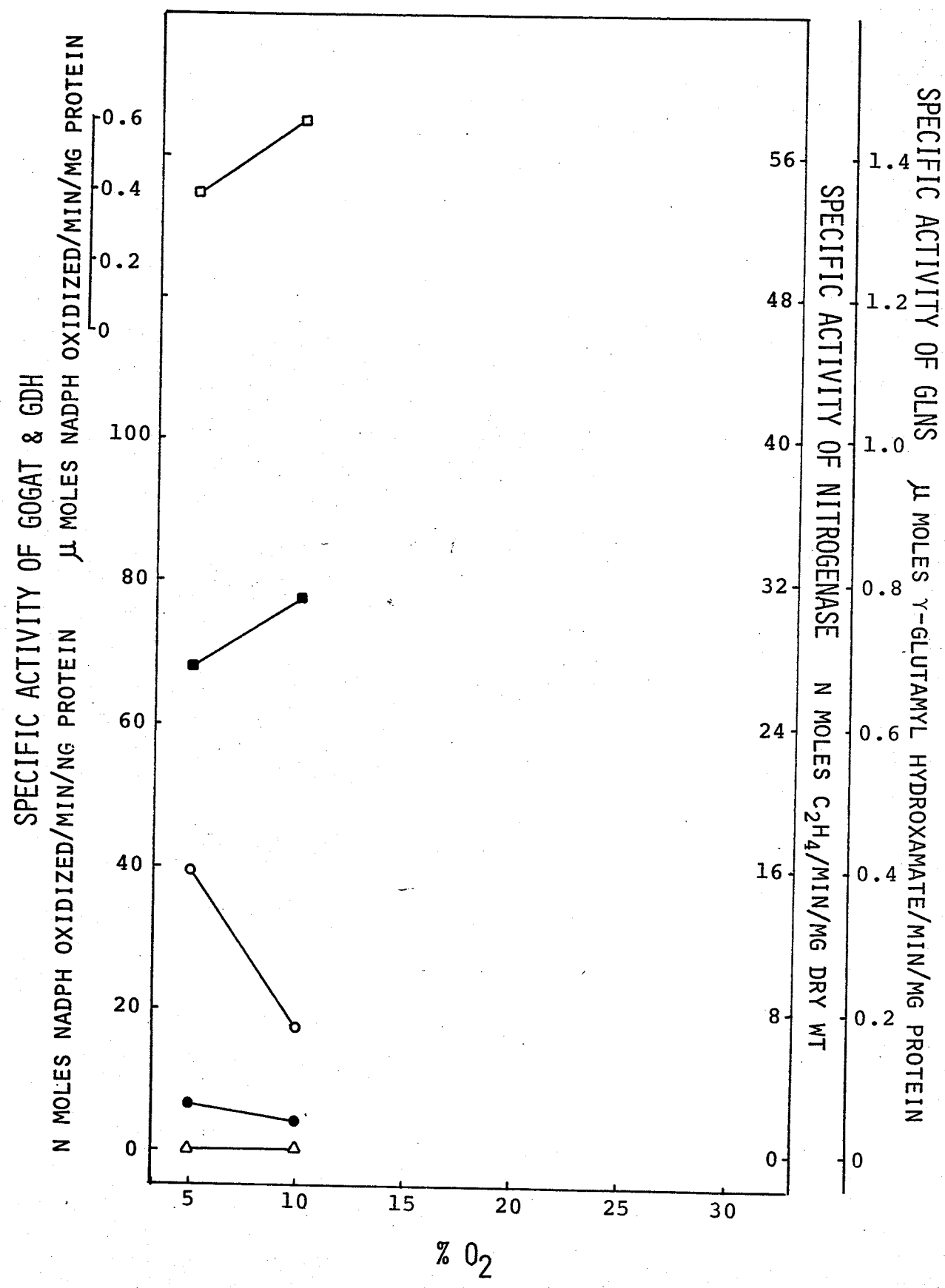

SPECIFIC ACTIVITY OF GOGAT & GDH N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
140
120
100
80
60
40
20
0
56
48
40
32
24
16
8
0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
30
% O_2

Fig. 20 Variation of enzymes activities with different concentrations of O_2 of continuous cultures of A. chroococcum at 30 mM NH_4^+ and at D = 0.22.




SPECIFIC ACTIVITY OF GOGAT & GDH
N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
μ MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
% O_2

VARIATION OF NITROGENASE, GOGAT, GLNS AND GDH WITH CHANGES IN THE NH_4^+ CONCENTRATION

(a) 5% O_2

At 5% O_2, as shown in Fig. 21, the nitrogenase rose to a peak level at 1 mM NH_4^+ and then dropped very sharply. No nitrogenase activity was detectable at 30 mM NH_4^+. The GOGAT dropped from 55 units under N_2-fixing conditions to half of this value at 20 mM NH_4^+; and then rose again sharply to about 70 units at 30 mM NH_4^+. The synthesis of the GLNS was apparently constitive throughout the range of NH_4^+ concentrations used. However, at higher NH_4^+ concentration most of the GLNS was in the adenylylated form. The rise of GDH was sharp but linear from N_2-fixing condition to a concentration of 20 mM NH_4^+ but thereafter the rise seemed almost unrestrained (the GDH level increased by 2.5 times between 20 and 30 mM NH_4^+).

(b) 10% O_2

Fig. 22 depicts the enzymatic changes accompanying changes in the NH_4^+ concentrations at 10% O_2 and at D = 0.22. The nitrogenase dropped rapidly as the NH_4^+ concentration increased in the same way as it did at 5% O_2, but apparently there was no peak at 1 mM NH_4^+. The GOGAT also dropped to a minimum at 20 mM NH_4^+ and rose again.

The synthesis of GLNS reached a peak at 4 mM NH_4^+ and then gradually decreased as the NH_4^+ concentration was increased. However the enzyme was not significantly repressed by NH_4^+ even at concentration as high as at 30 mM. It is obvious from Fig. 22 that the rise of GDH levels was biphasic. From concentrations of zero to 20 mM NH_4^+, the rise of GDH was linear but fairly slow. Above 20 mM NH_4^+, the rate of increase in GDH was almost explosive with the levels increasing 12 times between 20 mM and 30 mM NH_4^+.

(c) 20% O_2

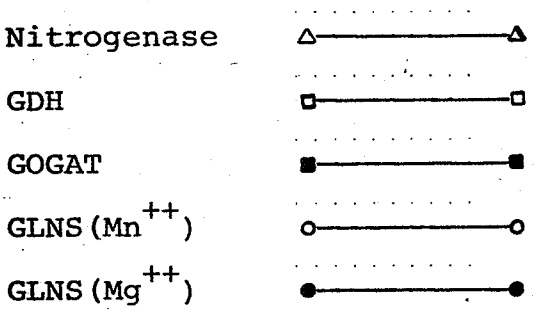
At 20% O_2, in Fig. 23, the nitrogenase, GOGAT and GLNS fluctuated at the lower NH_4^+ concentrations and then clearly decreased as the NH_4^+ concentrations increased. The rise of GDH was also linear, and about the same rate as at 10% O_2. O_2 toxicity (see The Effect of Oxygen)# prevented investigation at higher NH_4^+ concentration.

THE RELATION BETWEEN NITROGENASE LEVELS AND NH_4^+ CONCENTRATIONS UNDER RESTRICTED O_2 SUPPLY

It will be seen in Figs. 21 and 22 that at 5 and 10% O_2 the nitrogenase levels observed in the bacteria seemed to decrease "exponentially" with increase in the concentrations of NH_4^+ supply. A logarithmic plot of the nitrogenase levels against the NH_4^+ concentrations in Fig. 24 shows that this "exponential"drop was in fact exponential.

see page 11

Fig. 21 Variation of enzymes activities with different concentrations of NH_4^+ of continuous cultures of A. chroococcum at 5% O_2 and at D = 0.22.



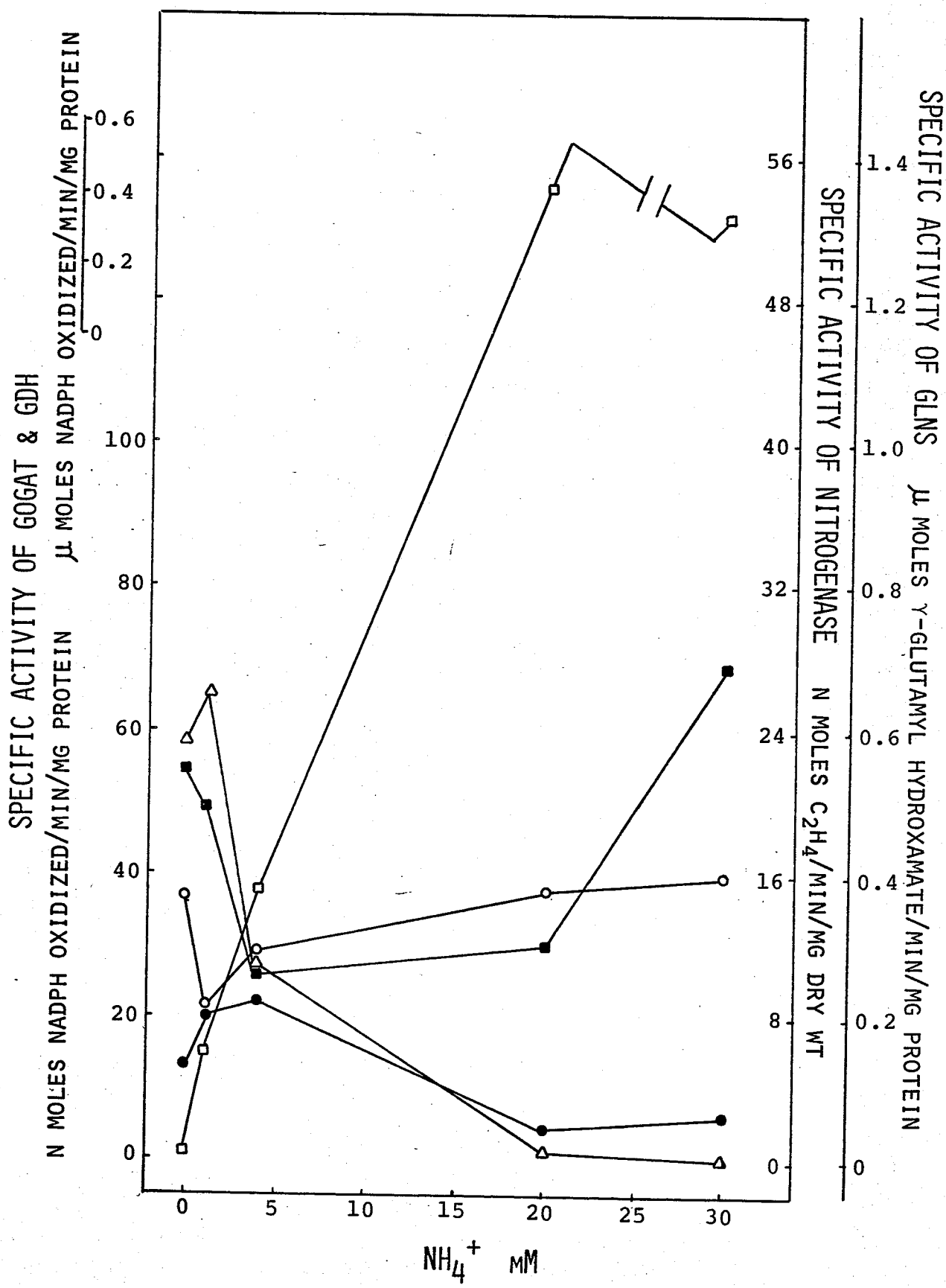
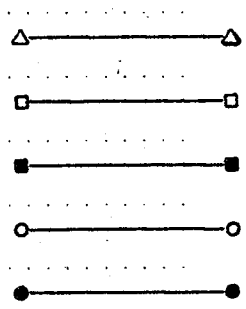

SPECIFIC ACTIVITY OF GOGAT & GDH
N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
μ MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
NH_4^+ mM
0
5
10
15
20
25
30
0
20
40
60
80
100
0
8
16
24
32
40
48
56
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
0.2
0.4
0.6

Fig. 22 Variation of enzymes activities with different concentrations of NH_4^+ of continuous cultures of A. chroococcum at 10% O_2 and at D = 0.22.



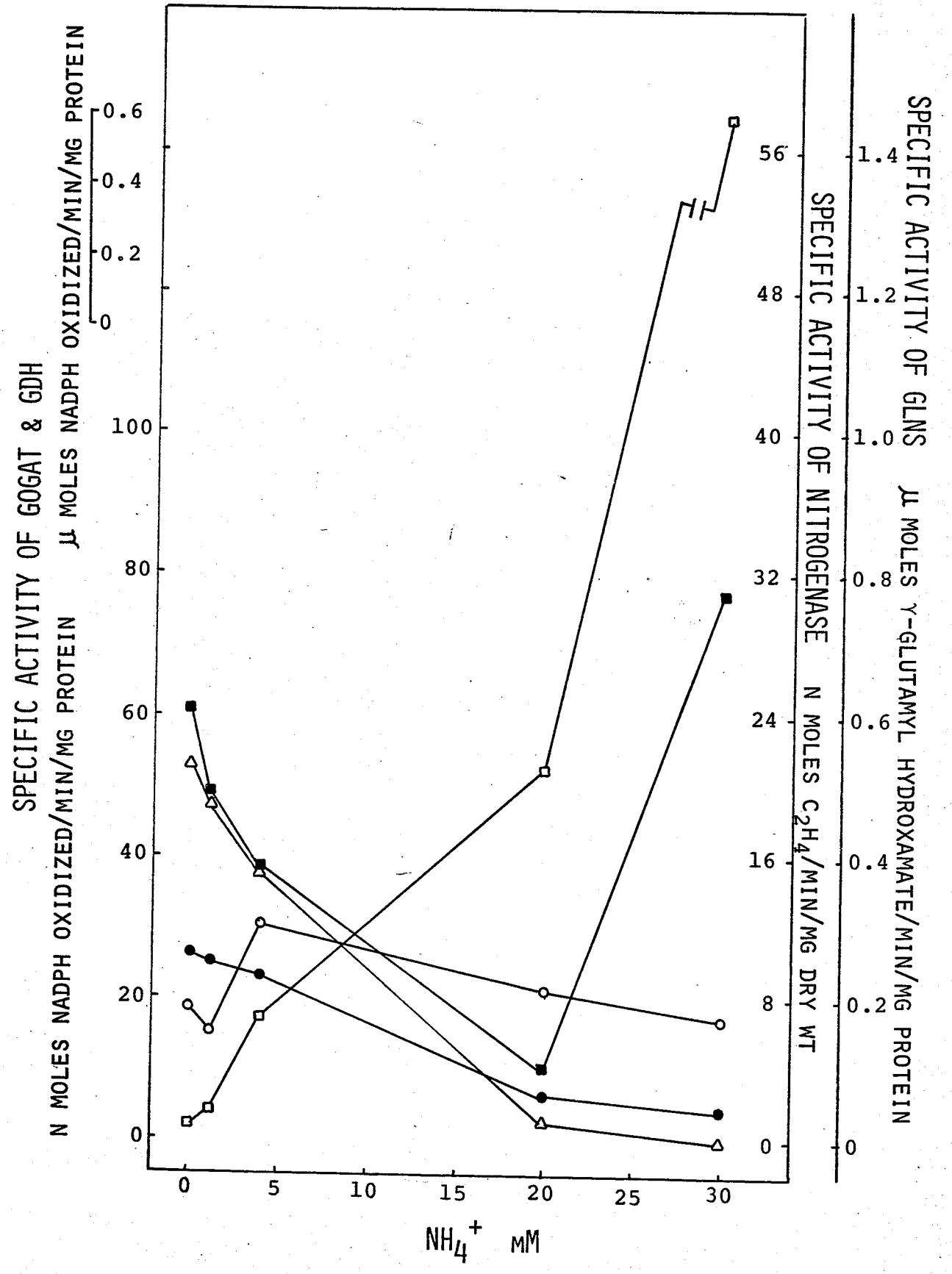
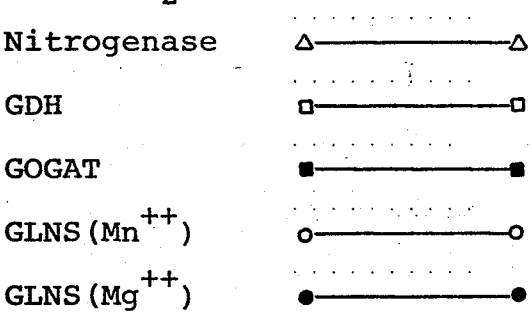

SPECIFIC ACTIVITY OF GOGAT & GDH
N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
μ MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE
N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS
μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
NH_4^+ mM
0
5
10
15
20
25
30
0.6
0.4
0.2
100
80
60
40
20
56
48
40
32
24
16
8
1.4
1.2
1.0
0.8
0.6
0.4
0.2

Fig. 23 Variation of enzymes activities with different concentrations of NH_4^+ of continuous cultures of A. chroococcum at 20% O_2 and at D = 0.22.



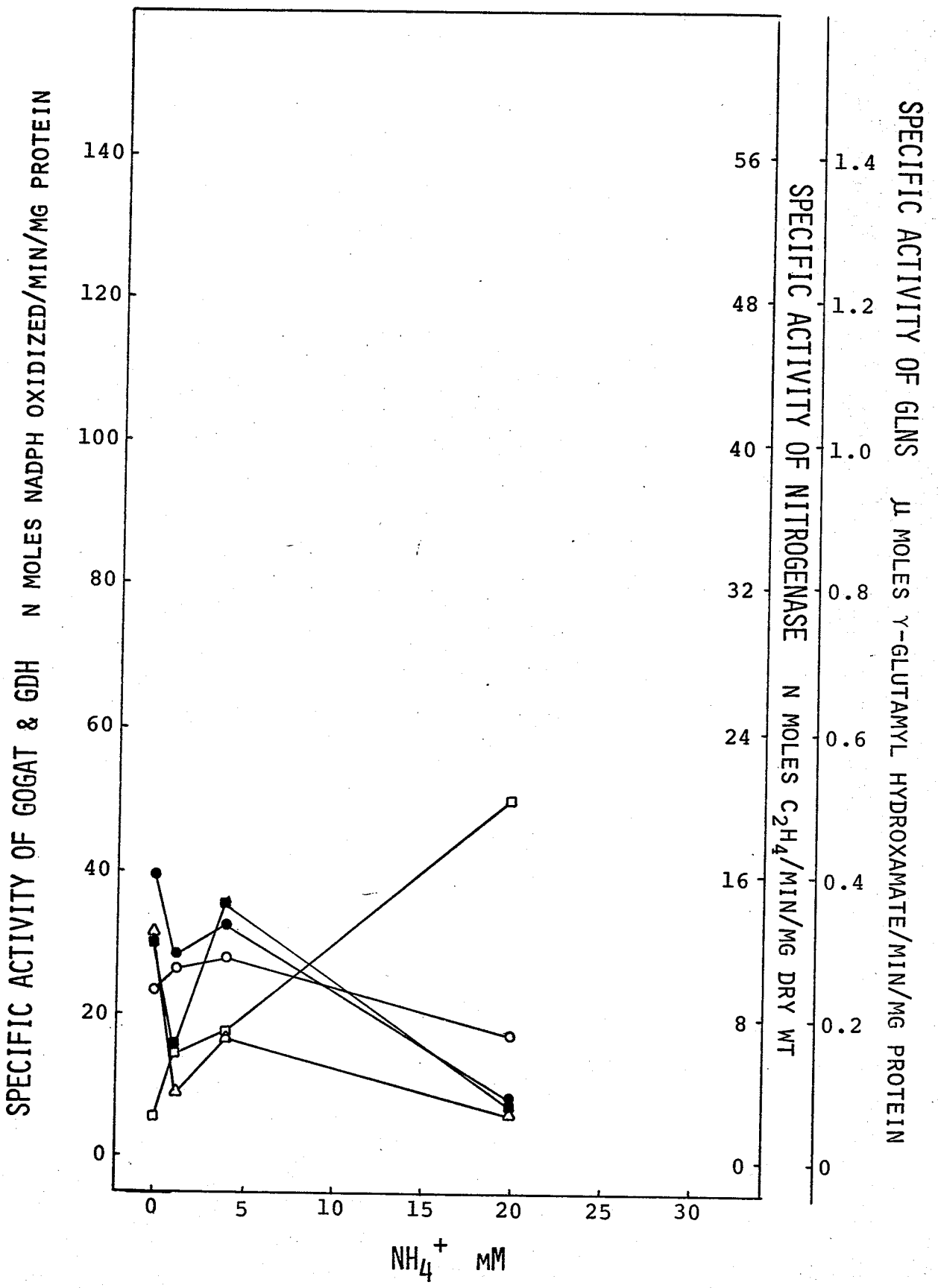

SPECIFIC ACTIVITY OF GOGAT & GDH N MOLES NADPH OXIDIZED/MIN/MG PROTEIN
SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
SPECIFIC ACTIVITY OF GLNS μ MOLES γ-GLUTAMYL HYDROXAMATE/MIN/MG PROTEIN
NH_4^+ mM
0
20
40
60
80
100
120
140
8
16
24
32
40
48
56
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
5
10
15
20
25
30

Fig. 24 The natural logarithmic plot of nitrogenase activity of continuous cultures of A. chroococcum against different concentrations of NH_4^+ at 5 and 10% O_2.

5% O_2 ——— o

10% O_2 ——— •

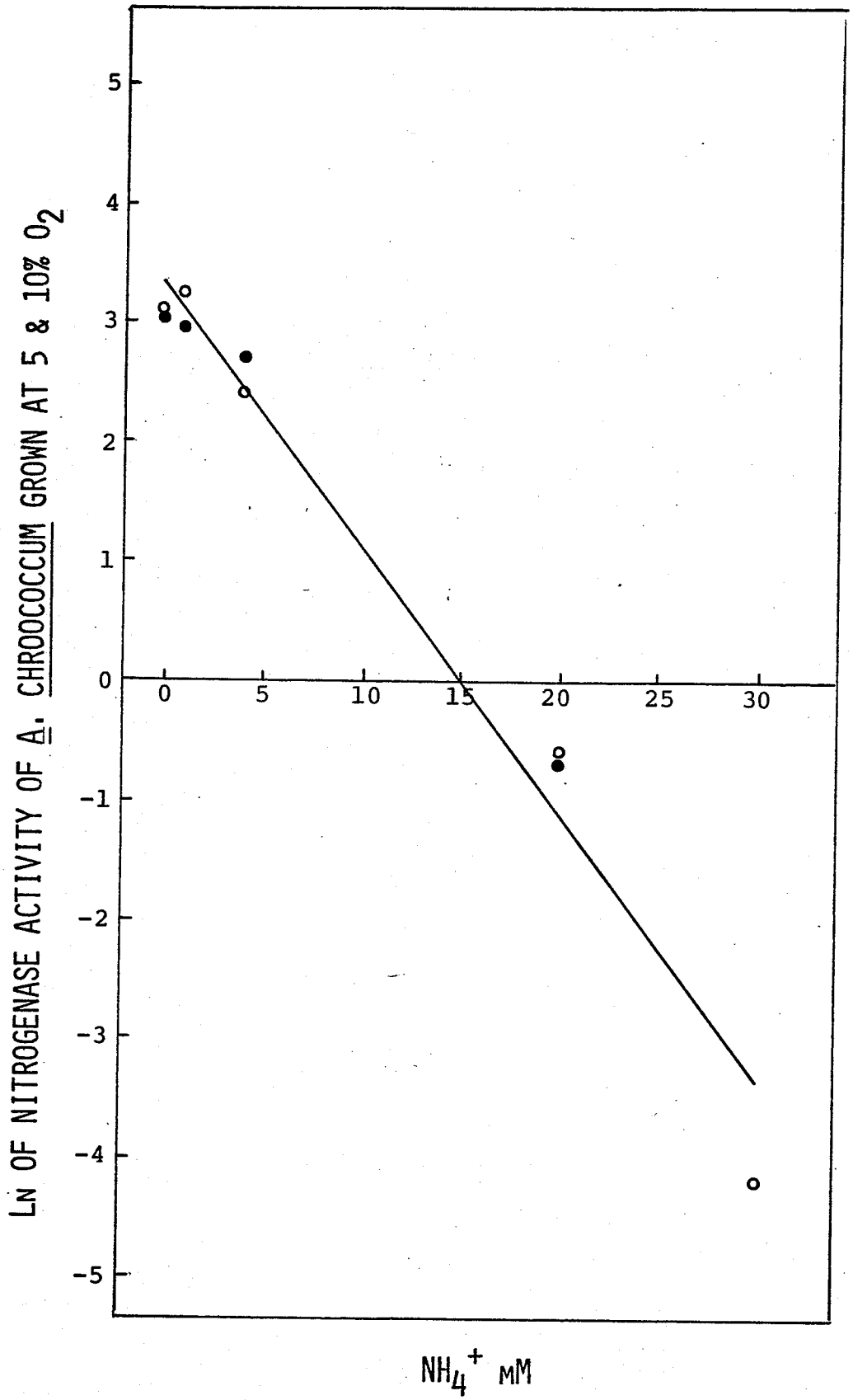
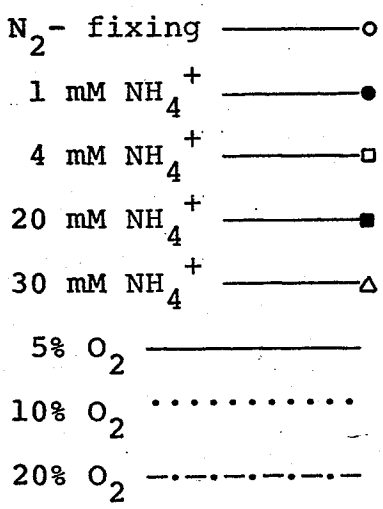
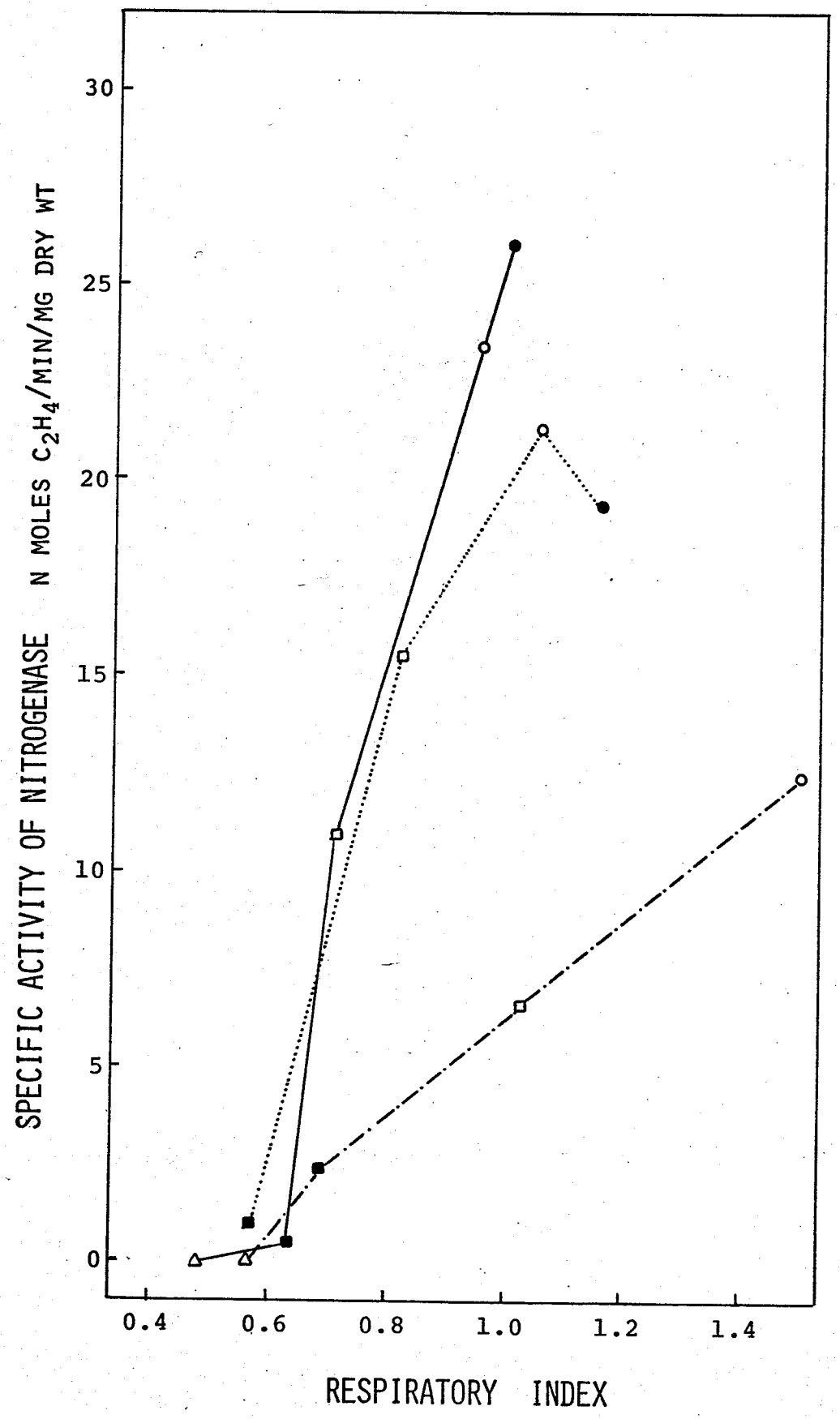

LN OF NITROGENASE ACTIVITY OF A. CHROOCOCCUM GROWN AT 5 & 10% O2
5
4
3
2
1
0
-1
-2
-3
-4
-5
0
5
10
15
20
25
30
NH4+ mM

Fig. 25 Variation of nitrogenase activity with Respiratory Index of continuous cultures of A. chroococcum at 5%, 10%, 20% O_2, at different concentrations of NH_4^+ and at D = 0.22.




SPECIFIC ACTIVITY OF NITROGENASE N MOLES C_2H_4/MIN/MG DRY WT
30
25
20
15
10
5
0
0.4
0.6
0.8
1.0
1.2
1.4
RESPIRATORY INDEX

D I S C U S S I O N

DISCUSSION

The growth of A. chroococcum was found to be excellent in continuous cultures stirred at 1750 r.p.m.. The rate of solution of O_2 from the air into a bacterial culture of this 200 ml volume was found to be considerably less than the rate of solution into water and is governed by complex factors not fully understood (Hine & Lees, 1976). Although the O_2 levels measured in all actively growing cultures were zero or very close to zero this does not necessarily mean that the cultures were starved from O_2 (c.f. Hine & Lees, 1976). From the CO_2 outputs, the biomass values and the high specific growth rates of the cultures, it is clear that the bacteria were not suffering from marked O_2-limitation when grown under the gas phase of air.

Actually, under N_2-fixation conditions and at 20% O_2 (air) the cultures were possibly under a slight O_2-limitation because of the stable biomass with increasing dilution rates (Fig. 6) and the slight increase of biomass with increasing O_2 (Fig. 7) (see The Basic Theory of Continuous Culture#; Tempest, 1970; and Hine & Lees, 1976). Moreover, the rise of the biomass with increasing concentrations of NH_4^+ (Fig. 7) indicated that the N_2-fixing cultures were under NH_4^+-limitation as well. Experimental results supporting this argument

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are: (i) the nitrogenase was not totally repressed even in cultures grown in 20 mM NH_4^+ (Figs. 22 & 23), and (ii) the nitrogenase levels increased with increasing dilution rates in N_2-fixing cultures and in cultures grown in low concentration of NH_4^+ in the media, while the GDH, GOGAT and GLNS remained relatively constant except at the washout points (Figs. 13, 14 and 15). Presumably, under N_2-fixing conditions the increase of the growth rates requires a corresponding increase of available nitrogen and this can be met only by increasing the nitrogenase content of the cells. It should be noted that at no time were the cultures carbon-limited; as Fig. 9 shows there were always some mannitol residual in every culture (c.f. Hine & Lees, 1976).

Dalton & Postgate (1969b) observed an ambient O_2 concentration of about 20 μM in a N_2-fixing A. chroococcum culture. However, this level of ambient O_2 was not detected in any of the actively growing cultures reported here. Indeed, whenever the O_2-meter registered a level of more than 10 μM O_2 in the culture, washout of the cells ensued. The absence of the appreciable amounts of dissolved O_2 in the N_2-fixing and other NH_4^+ grown cultures could be explained by the findings of Hine & Lees (1976). These findings also explained the apparent discrepancies between the results obtained in this laboratory and those obtained at University of Sussex (c.f. Dalton & Postgate, 1969b). The O_2 in passage from the gas phase to the cells must traverse the gas-liquid and liquid-cell interfaces, both of which can

offer great resistance to any gas transfer. If the liquid-cell interface is offering resistance, as it seems to be in mild stirring of the liquid phase, the dissolved O_2 would have to build up an appreciable concentration gradient before reaching the cells; this would consequently be measured by the O_2-electrode as ambient dissolved O_2. It was noticed during the present investigation that if the vigorous stirring of an actively growing culture was momentarily stopped and subsequently resumed, the O_2 level in the culture would unfailingly shoot up, then gradually subside. (This fact, although repeatedly observed is not reported in the Results section in this thesis.) Presumably, this phenomenon is due to the delay between the time of dissolved O_2 build up and the time of O_2 penetration of the liquid-cell interface. On the other hand, if both interfaces are disturbed, as it seems to be in vortex stirring of the liquid phase, the dissolved O_2 would be in direct contact with the cells. Provided the cells have a high O_2 consumption rate, as Azotobacter does, the dissolved O_2 at vortex stirring would be used up and the O_2-electrode would measure zero O_2. Indeed, when the O_2 consumption rate of the cultures was reduced (cultures grown in high concentrations of NH_4^+) or pushed to the limit (N_2-fixing cultures grown at 50% O_2) a rise of O_2 level in the culture was observed. However, this rise of O_2 was followed by washing out of the culture. In recent private conversations, Postgate and Lees agreed that the growth conditions of cells in "ordinarily stirred" cultures and in

"vortex-stirred" cultures are probably so different that direct biochemical comparisons between cultures grown under the two conditions of stirring may be misleading.

In contrast to the previous observation (see The Effect of Oxygen)# NH_4^+ rendered the A. chroococcum more sensitive to dissolved O_2 than it was under N_2-fixing conditions. It can be seen in Table 1 that a N_2-fixing culture at D = 0.22 could withstand up to 50% O_2, while a culture growing in 30 mM NH_4^+ at the same dilution could not even survive at 20 % O_2. Even more strikingly, a culture growing in 50 mM NH_4^+ was washed out at O_2 concentration as low as 5%. The inability of NH_4^+-grown cultures to grow at higher concentrations of O_2 was at first difficult to accept since it has been generally agreed that N_2-fixing Azotobacter cultures are more O_2 sensitive than the NH_4^+ assimilating ones. Furthermore, the extremely O_2 sensitive nature of the enzyme nitrogenase is well established. Therefore, Clostridium and Klebsiella (except K. pneumoniae M5al, Hill, 1976) can fix N_2 only when grown in anaerobic or microaerophilic conditions. Azotobacter which is essentially strictly aerobic, has evolved complex protective devices such as respiratory protection and conformational protection to keep the nitrogenase functional. However, a close look at the O_2 consumption rates, measured in terms of CO_2 outputs (Fig. 11) reveals that the cultures grown in 20 and 30 mM NH_4^+ were unable to produce as much CO_2 as the N_2-fixing

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cultures or those fed with 1 and 4 mM NH_4^+, despite the fact that the biomass of the cultures at the higher NH_4^+ concentrations had almost twice the biomass of the cultures at the lower NH_4^+ concentration. Their Respiratory Index values (Fig. 11) clearly show the decreasing order of the ability of the cultures to produce CO_2 with increasing concentrations of NH_4^+ in the media. The only reverse in the order is observed with the cultures fixing N_2 and those fed with 1 mM NH_4^+ at 5% and 10% O_2. Hine & Lees (1976) observed an apparent levelling off of the Respiratory Index at about 0.11 when the gas phase above the culture contained about 20% O_2. However, Fig. 11 shows that there was still a slight fall-off of the Respiratory Index values between 20% O_2 and 5% O_2. The respiratory index values of cultures grown in 20 mM and 30 mM NH_4^+ were virtually stable throughout the range of O_2 concentrations used. Since under these conditions the levels of nitrogenase in these cultures were very low, their Respiratory Index values would probably represent the minimum CO_2 produced in cellular growth and maintenance alone. Figs. 16 and 17 show that the nitrogenase activities at 5% and 10% O_2 were high and that there was no sign of any O_2 poisoning at all. Therefore the difference of the Respiratory Index values between the N_2-fixing and the 30 mM NH_4^+-grown cultures at 5% O_2 probably represents the amount of respiration necessary for the production of energy and materials for N_2-fixation. Any Respiratory Index values larger than that at 5% O_2 concentrations and under

N_2-fixation conditions would be the extra respiration probably due to respiratory protection. Further increase of O_2 concentrations in N_2-fixing cultures sees an increase of respiratory index values but a rapid decline of nitrogenase to a lower level (Fig. 16). Similar effects of O_2 are also seen in cultures growing in 1 mM NH_4^+ (Fig. 17). Apparently, the respiratory protection is not effective at all to protect the nitrogenase against O_2 inhibition. These results nevertheless raise a question whether the respiratory protection is particularly for the nitrogenase or for the cells as a whole. So far, no information has been obtained to pin-point the actual function of the respiratory protection. Recently, Fridovich and his co-researcher (Kellogg & Fridovich, 1975) suggested that the powerful oxidant, singlet O_2 (O_2*) produced by:

$$H_2O_2 + O_2^- \longrightarrow OH^- + OH. + O_2*$$

would be the probable cause for O_2 toxicity. And, it has been established that the superoxide radical O_2^-, which takes part in making singlet O_2, is produced by many spontaneous and biological reactions (Fridovich, 1974). In view of this, it is not unreasonable to assume that the respiratory protection is a general protective mechanism against "O_2 poisoning" and the nitrogenase happens to be a highly "O_2-sensitive" enzyme. The work presented in this thesis however was not designed to prove or disprove this point.

Drozd et al., (1972) found the GDH and GOGAT contents were almost the same in A. chroococcum growing under N_2-fixing condition in continuous cultures. They also observed no substantial difference in the ratio of these enzymes even when the cultures were grown at the same dilution rate in the presence of fixed N such as NH_4^+ and NO_3^-. However, the results observed in this present thesis are quite different. Under N_2-fixation conditions (Fig. 13), the GOGAT level was high and comparable to that observed by Drozd et al., (1972) but in our cultures the GDH was barely detectable. The GLNS was present at high level and was mainly in the deadenylylated form. In line with the findings of Meers et al. (1970) in K. (Aerobacter) aerogenes the GOGAT/GLNS couple was apparently operative at low NH_4^+ while GDH was not.

The effects of O_2 levels on these enzymes was striking. Under N_2-fixation conditions and at 5% and 10% O_2 there was a change in GLNS from the predominantly adenylylated form to the deadenylylated form (Fig. 16). At higher O_2 concentrations, the deadenylylated form prevailed, yet, there was no repression of the total biosynthesis of GLNS by the O_2. However, the GOGAT which is not known for its O_2 sensitivity was seen to decrease with increasing O_2 levels. This finding is remarkable in view of the work of Meers et al., (1970), which suggested that the GLNS should work best when coupled with GOGAT. Therefore, a steady level of GLNS should require a steady level of GOGAT in order to maintain a constant

supply of L-glutamate while the GDH activity is denied to the organisms. This atypical relation of the GOGAT/GLNS couple was also observed with increasing O_2 concentration in 1 mM NH_4^+-grown cultures (Fig. 17). Figs. 16 and 17 also show a reverse relation between nitrogenase and the deadenylylated GLNS with increasing O_2. It appears that if the deadenylylated GLNS can derepress nitrogenase as it does in K. aerogenes (Streicher et al., 1974), its effect in Azotobacter is overshadowed by the overall effect of O_2 concentration.

The response of these enzymes (GDH, GOGAT and GLNS) toward O_2 is markedly affected by the amount of NH_4^+ present. The change of O_2 concentrations from 5% to 10% O_2 repressed the GDH, GOGAT and GLNS in cells growing in 20 mM NH_4^+ (Fig. 19). The same change of O_2 applied to cultures fed with 30 mM NH_4^+ resulted in derepression of the GDH and GOGAT (Fig. 20). Apparently, therefore, the effect of NH_4^+ is dominant over the effect of O_2 and thus the effect of O_2 and NH_4^+ and the level of deadenylylated GLNS on the enzymatic pattern of Azotobacter are in the order: $NH_4^+ > O_2 >$ deadenylylated GLNS.

The effect of NH_4^+ on the nitrogenase, GOGAT, GLNS and GDH in Azotobacter is shown in Figs. 21-23. Under N_2-fixing conditions or in the presence of 1 mM NH_4^+ the nitrogenase provided the cells with the essential NH_4^+ that was incorp-

orated by the GOGAT/GKNS couple. When the NH_4^+ was raised to 4 mM, the activity of the nitrogenase and the GOGAT/GLNS couple was heavily impaired. What the NH_4^+ apparently did was adenylylate the GLNS already present, not repress its biosynthesis. On the other hand, the rise of GDH was directly proportional to the increase of NH_4^+, indicating the gradual take-over of NH_4^+ assimilation by the GDH system. Further increase of NH_4^+ saw an exponential decrease of the nitrogenase, (Figs. 21,22 and 24). From 20 to 30 mM NH_4^+, the GOGAT and GDH but not GLNS increased dramatically. A similar finding was also observed in E. coli growing at NH_4^+-limitation with a high dilution rate at which nevertheless a high concentration of extracellular NH_4^+ was present (Senior, 1975).

At 20-30 mM NH_4^+ (Figs. 21 and 22), the rise in GOGAT content is possibly unimportant in terms of NH_4^+ assimilation, because the L-glutamate that the GOGAT makes may well be swamped by the large amount of L-glutamate produced by the GDH present. Actually, the combined effect of both GDH and GOGAT may well be to promote the reverse reaction catalyzed by the GDH i.e. the formation of α-ketoglutarate and NH_4^+ from L-glutamate. In view of this, it is difficult to assess the biosynthetic role of either enzyme when the NH_4^+ is present in high concentration (20 - 30 mM). Indeed, the GDH in E. coli (Senior, 1975) can play both biosynthetic and degradative roles depending on the situation. Figs. 21 and 22 show that the GDH increase was biphasic with a dividing mark

at 20 mM NH_4^+. It looks as though the synthesis of GDH at low concentrations of NH_4^+ was primarily for biosynthetic purposes, because the nitrogenase and the GOGAT/GLNS couple were lowering their levels and the GDH rose linearly with increasing NH_4^+ concentration. The rapid rise of the GDH and GOGAT in the presence of more than 20 mM NH_4^+ was presumably of degradative nature.

In E. coli (Senior, 1975, Fig. 1) at NH_4^+-limitation and growing at high dilution rates, the extracellular NH_4^+ rose rapidly and at the same rate with the rise of GOGAT. This suggested that the combined action of GDH and GOGAT somehow reduced the NH_4^+ uptake by the cells. The cells of A. chroococcum growing at high concentration of NH_4^+ (>20 mM) would be suffering a large influx of NH_4^+, which consequently would lower the respiratory activity of the cells (Fig. 11) and accentuate the inhibitory effect of O_2. To counteract the influx of NH_4^+, the cells would require an NH_4^+ pump, pumping the intracellular NH_4^+ out. This could be achieved by producing large amount of GDH and GOGAT. The L-glutamate produced by the GOGAT would upset the reaction equilibrium of GDH so that the net effect would be the breakdown of L-glutamate and producing α-ketoglutarate and NH_4^+. Therefore, the combined action of GDH and GOGAT at high concentration of NH_4^+ would probably be a protective mechanism against the buildup of excessive intracellular NH_4^+. The inability of the culture grown at 30 mM NH_4^+ and at 10% O_2 to survive

higher concentrations of O_2 is an indication that the cells had already stretched to the highest rate of GDH biosynthesis (Fig. 20). The death of the culture fed with 50 mM NH_4^+ at 5% O_2 may well be explained by this hypothesis.

The regulation of GOGAT, GLNS and GDH in A. chroococcum is quite different from that observed in E. coli (Senior, 1975) and in K. (Aerobacter) aerogenes (Meers et al., 1970b). At NH_4^+-limitation, the K. aerogenes and A. chroococcum use the GOGAT/GLNS couple while the E. coli maintains its GLNS in the most active state. As the surrounding NH_4^+ becomes more readily available, the GDHs of A. chroococcum and E. coli rise accordingly. The GDH of K. aerogenes, however remains low until the NH_4^+ is plentiful. When NH_4^+ is supplied in larger amounts the GOGATs of A. chroococcum and E. coli suddenly shoot up to a high value, but not the GOGAT of K. aerogenes. However, under this condition, both GLNSs of E. coli and K. aerogenes are totally repressed. The biosynthesis of GLNS in A. chroococcum is not significantly repressed, instead, the enzyme already present is inactivated by adenylylation.

The work in this thesis does not support nor deny the findings of Magasanik and his co-researchers (Streicher et al., 1974) in K. aerogenes. In this strain of A. chroococcum the GLNS behaved as if a constitutive enzyme, and there was always a small amount of deadenylylated enzyme present even in the

most repressed state. It is quite possible that only a very small amount of deadenylylated GLNS is enough to initiate the repression or derepression of the synthesis of nitrogenase, GOGAT or GDH. Nevertheless, the O_2 and NH_4^+ can take part directly, or indirectly in the regulation of these enzymes. Moreover, if the deadenylylated GLNS had any control of the regulation of these enzymes, its effect is less significant than that of O_2 and NH_4^+ in this strain of A. chroococcum.

The patterns of enzyme variation in A. chroococcum, in response to variations in NH_4^+ concentration, dilution rate, and O_2 tension, cannot be directly compared with previous results obtained in this field. Drozd et al. (1972) watched the decrease in nitrogenase in A. chroococcum cultures after the addition of ammonium succinate, but their experiments lasted for four hours. The results were unequivocal and showed an immediate effect of externally added NH_4^+ on the nitrogenase levels in the bacteria. The present work is based, not on immediate effects, but on the long-term overall effect of (say) NH_4^+ in stabilizing the level of (say) nitrogenase within a new steady-state population of cells that establishes itself under the new environmental conditions. This new steady-state population may represent a new selection of mutants (Gordon & Brill, 1972). This point has not been investigated because it is not immediately germane to the experimental results.

An example of the new type of finding embodied in the present work is illustrated in Fig. 24 which shows that the steady-state level of NH_4^+ in a culture establishes a level of nitrogenase in the bacteria that is logarithmically related to the NH_4^+ concentration. Mathematically this means that

$$-\frac{d(\text{nitrogenase})}{d(NH_4^+)} = K(\text{nitrogenase})$$

i.e. the nitrogenase concentration, K, is directly related to the rate of change of nitrogenase concentration and inversely to the rate of change of NH_4^+ concentration. The biochemical implications of this fact are however beyond the scope of this work. Although it should be pointed out that if the interaction of small amount of O_2 leaking into the cell with nitrogenase in the cell produces the O_2^- that destroys the nitrogenase the explanations of many results would be very much simplified.

The fall in Respiratory Index with increasing NH_4^+ concentrations is to be expected since, if the organism is not involved in producing biologically negotiable energy to drive the N_2-fixation reactions, the less energy it will need to produce a given weight of cells and so the less CO_2 it will evolve in producing the cells. What is surprising however (Fig. 25) is the large increase in nitrogenase when the cells are grown under low or zero NH_4^+ concentrations with low concentrations of O_2 in the gas phase. At any given R.I. value the cells are growing with the same "efficiency" in that they

are burning the same amount of mannitol to CO_2 to achieve the same amount of cell growth, yet at an R.I. of 0.1 the nitrogenase content is related inversely to the O_2 concentration in the gas supply. The cells clearly do not need more nitrogenase to grow at the lower O_2 concentrations nor do they use more because if they did the extra energy supply would raise the R.I. above 0.1. The only explanation appears to be that at the higher O_2 concentrations, the nitrogenase is "deactivated" independently of any "respiratory protection" or any other sort of protection. It appears from other work going on in this laboratory (Buchanan & Lees, in preparation) that this "deactivation" is probably due to the O_2^- anion (or singlet oxygen O_2*) because at higher O_2 concentrations, A. chroococcum manufactures more superoxide dismutase in an apparent effort to rid itself of the unwanted O_2^- or O_2*.

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