THESIS

This is to certify that the

thesis entitled
The Regulation of Nitrogen Fixation
in Azotobacter vinelandii:
A Biological Systems Approach

presented by
Thomas H. Merriewether

has been accepted towards fulfillment
of the requirements for

Master of Science degree in Microbiology

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Date Feb. 22, 1980

THE REGULATION OF NITROGEN FIXATION IN *AZOTOBACTER VINELANDII*: A BIOLOGICAL SYSTEMS APPROACH

By

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology
and Public Health

1980

ABSTRACT

THE REGULATION OF NITROGEN FIXATION

IN AZOTOBACTER VINELANDII:

A BIOLOGICAL SYSTEMS APPROACH

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A comprehensive study of the dynamics of nitrogen metabolism in A. vinelandii was performed with emphasis on nitrogen fixation. The key targets of the study were nitrogenase, glutamine synthetase, glutamate dehydrogenase, cAMP pools, and ammonium uptake.

In chemostatic cultures of A. vinelandii, the existence of late, inducible ammonia translocases was confirmed as had been first observed by Kleiner (25). Coincidence was found between the rise in late ammonium translocase activity and glutamine synthetase indicating a possible linkage between the two.

With regard to ammonia assimilation, A. vinelandii was found to modulate or latch between the glutamine synthetase system and glutamate dehydrogenase depending on the level of exogenous ammonia. The effect of this modulation was found to be complex, resulting in

a non-linear shift in growth rates and, therefore, ammonia assimilatory efficiencies.

The system(s) regulating nitrogenase activity was found to vary in dynamic behavior depending on the mode of ammonia addition. In chemostatic cultures, gradient addition of ammonia resulted in a stable, exponential decline in nitrogenase activity whereas a rapid pulse of ammonia resulted in unstable, oscillatory behavior. There was, also, evidence of "tight" coupling between nitrogenase, glutamine synthetase, and adenyl cyclase activities.

The dynamic responses of the components under study were approximated with algebraic polynomials which will serve as a basic model for future studies.

DEDICATION

To my loving parents

ACKNOWLEDGEMENTS

I wish to express my most sincerest gratitude to Dr. Harold Sadoff for his guidance and support of this rather unorthodox approach to an understanding of biological nitrogen fixation.

I am also greatly indebted to the support and aid given me by the people at Difco Laboratories. Special thanks to Mrs. Shirley Huick and Mr. Verland Wheat.

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LITERATURE REVIEW

Azotobacter vinelandii

The genus, *Azotobacter*, is of the order, *Eubacteriales*, and the family, *Azotobacteraceae* (23).

The family, *Azotobacteraceae*, contains the genera *Azotobacter*, *Beijerinckia*, *Azomonas*, and *Derxia* (23). Common characteristics of the genera of this family are the ability to fix atmospheric nitrogen, strictly aerobic respiration, marked pleiomorphism, and the production of copious polysaccharide slime (23). Of the genera, all but *Azomonas* produce thick-walled resting bodies known as cysts. Cysts tend to be more resistant to adverse environmental conditions than vegetative cells and, except for heat resistance, are functionally analogous to the endospores formed by members of the family, *Bacillaceae* (22,23,32, 41).

Species of the genus, *Azotobacter*, are characterized as being large, ovoid cells 2um in diameter, though markedly pleiomorphic, producing thick-walled cysts, and producing prodigious quantities of capsular slime (23). Members of this genus also fix 10-15 mg nitrogen

per gram of carbohydrate consumed (23). Azotobacter vinelandii and Azotobacter paspali also produce a green, fluorescent pigment (23). The genus, Azotobacter, contains four species, A. chroococcum, A. vinelandii, A. beijerinckii, and A. paspali (23). A. chroococcum is the type species.

A. vinelandii was first isolated from the soil of Vineland, New Jersey by Lipman in 1903 (23). The organism is motile by peritrichous flagella and produces copious amounts of polyglucuronic acid slime (23). A. vinelandii is the only member of the genus capable of utilizing rhamnose as a carbon source (23).

Biological Nitrogen Fixation

Biological nitrogen fixation is the sequence of enzyme catalyzed events whereby molecular nitrogen is reduced to ammonia. The biological reduction of molecular nitrogen is an energy-dependent process, which utilizes ATP in the majority of cases (12).

Biological nitrogen fixation was observed in 1888 by Beijerinck who successfully isolated a bacterium from root nodules of a leguminous plant. The bacterium was shown to be capable of direct assimilation of molecular nitrogen by a, hitherto, unknown pathway (12). Winogradsky, in 1893, isolated the first, free-living,

nitrogen-fixing bacterium from an anaerobic culture. This organism, Clostridium pasteurianum, was also shown to have the capacity for the direct assimilation of molecular nitrogen.

To date, nitrogen fixation is a phenomenon which only occurs among bacteria and cyanobacteria (12). However, within the kingdom of the prokaryotes there is wide diversity among the physiological types which fix nitrogen (4,9,12). These include members of the families, Bacillaceae, Azotobacteraceae, Athiorhodaceae, Pseudomonadaceae, and Enterobacteriaceae (4,9,12). Prokaryotes fixing nitrogen may be autotrophic or heterotrophic, aquatic or terrestrial, aerobic or anaerobic, and free-living or in symbiosis with higher organisms. One can gain an increased sense of the diversity among nitrogen fixing organisms by an examination of the co-symbionts which associate with symbiotic nitrogen-fixers. For example, Citrobacter spp. in the termite gut, Rhizobium spp. associated with the root systems of leguminous plants, lichens (cyanobacteria/fungi), and a reported symbiosis between a species of Klebsiella and the gut of New Guinea natives (4,9). In addition, it has been noted that among the photosynthetic bacteria examined, the number having some nitrogen fixing capacity are in the majority (4).

Nitrogen fixation provides a significant fraction of the biologically available nitrogen on earth (9). Current estimates

indicate that biological nitrogen fixation contributes 150 million metric tons of ammonia per annum to the biosphere (9).

Nitrogenase is the enzyme complex containing the active site for dinitrogen reduction. Among all nitrogen fixing organisms studied, nitrogenase is the only entity responsible for nitrogen reduction. Nitrogenase is not only ubiquitous among nitrogen fixing organisms but possess striking structural and functional homology on an interspecies basis (4,9,12,13,14,47,49).

As stated above, nitrogen fixing organisms can be superficially classified as being free-living or symbiotic with higher organisms. Free-living species may exist independent of higher organisms. Symbiotic nitrogen fixing organisms, on the other hand, fix nitrogen inefficiently or not at all when disassociated from their co-symbionts. This is the case for members of the genus Rhizobium, which form symbioses with agriculturally important legumes (9). An interesting feature of the relationship is host-bacterium specificity (9). Some members of the genus, Rhizobium trifolii for example, will only enter into symbioses with specific legume species while others of the genus enter into non-specific symbioses with a wide range of legume species (eg. the cowpea-Rhizobium group). With the species R. trifolii, there seems to be a specific lectin binding agent on clover root hairs which usually selects R. trifolii as a co-symbiont (9). The symbiotic interaction often leads to the production of root nodules which

contain the bacteria (bacteroids) encapsulated within a membranous sac (9).

Some cyanobacteria possess specialized structures, heterocysts, which are the sites of nitrogen fixation (5). Although molecular hydrogen is known to be an inhibitor of nitrogen fixation, some cyanobacteria utilize hydrogen as a source of reducing power for dinitrogen reduction (5,17).

Nitrogenase

Nitrogenase is the enzyme complex catalyzing the biological reduction of dinitrogen. The term "nitrogenase" is somewhat of a misnomer in that the enzyme of all known nitrogen fixing organisms exists as two, distinct, protein entities or "components". Component I, also known as the molybdenum-iron protein and molybdoferredoxin and "nitrogenase", contains the active site for the binding and reduction of molecular nitrogen. Component I has a combined molecular weight in the range 227-270 kilodaltons (Kd), and exists as a tetramer of two, distinct subunit types. Molybdoferredoxin possesses 34-36 gram atoms of Fe, 26-28 gram atoms of labile S, and 2 gram atoms of Mo (4,9,12,13,47,49). The active site contains the molybdenum and is located in a small, dissociable polypeptide cofactor similar to that

of nitrate reductase and xanthine oxidase (36). Recent studies have indicated that molybdenum can be replaced by vanadium and tungsten, with some detectable dinitrogen reduction activity in a few cases (10,37). Component II, also known as the iron-sulfur protein and azoferredoxin and nitrogenase reductase, serves as the electron carrier to the active site of molybdoferredoxin (4,9,14,35,45,47,54). Azoferredoxin has an average molecular weight of 27-33 Kd, and exists as a dimeric enzyme of identical subunits (4,9,12,13,14,47). Recent studies on molybdoferredoxin and azoferredoxin have placed them in the ferredoxin class, hence the names component I and component II are now in disuse.

Both molybdoferredoxin and azoferredoxin are extremely oxygen labile. The exposure of purified azoferredoxin to air for five minutes results in complete loss of activity, and similar treatment of molybdoferredoxin results in a fifty-percent loss of its activity (27,45). Extreme oxygen lability is a property common to all nitrogenases thus far examined (4,9,12). The biological fixation of nitrogen has been assessed as being an essentially anaerobic process (4,9,12,45,54). This presents a special problem to aerobic nitrogen fixing organisms for these organisms must carry out normal respiration and anaerobic nitrogen fixation simultaneously. They must, therefore, possess mechanisms which protect the nitrogenase system. Azotobacter vinelandii is thought to protect its nitrogenase system

via two mechanisms. The first is made manifest by the organism's extremely high respiration rate (54). Studies on the respiratory mechanisms of A. vinelandii have measured its $Q(O_2)$ at 3,300 $ul(O_2)$ per milligram of protein (27,54). The respiratory cytochrome system of A. vinelandii has been shown to be tripartite (54). Two of the branches have coupling sites for oxidative phosphorylation. A third branch, cytochrome b_1 to cytochrome a_2, has no coupling site but has been shown to accomodate high electron flux during nitrogen fixation (54). It has been hypothesized that this "fast branch" of the cytochrome system serves as an oxygen scavenger, maintaining an intracellular $P(O_2)$ within limits tolerable for continued nitrogen fixation (54). The second method by which A. vinelandii may protect its nitrogenase system is termed "switch off" (16,27,54). Under conditions where normal respiration is inhibited or the intracellular $P(O_2)$ exceeds tolerable limits nitrogenase, via an unspecified mechanism, undergoes a conformational change (27,54). The change in conformation results in an inactive but relatively oxygen insensitive nitrogenase complex (16,27,54). Rhizobia are thought to protect the nitrogenase complex by yet another mechanism. In the rhizobium-legume symbiosis leghaemoglobin is produced synergistically (2). Leghaemoglobin is the product of haeme, synthesized by the rhizobia and a globin protein, synthesized by the plant. Leghaemoglobin binds oxygen in a manner

analogous to haemoglobin, thus reducing the $P(O_2)$ experienced by the bacteroids under conditions of high oxygen flux (2).

Previous attempts at characterization of the physical properties of the nitrogenase components were not entirely successful (45). Contamination of preparations by exogenous proteins (eg. ferredoxin) led to inconclusive spectral data (54). It was not until a feasible purification protocol had been developed that such physical characterization became possible. In 1973, Shah and Brill (45) developed a simple protocol for the complete separation and purification of the nitrogenase components. Clear separation of the two components was achieved on a DEAE cellulose column using a stepwise elution procedure. The molybdoferredoxin, by virtue of its heat stability, was further purified from contaminating proteins by differential denaturation. Final purification of each component was achieved via anaerobic polyacrylamide gel electrophoresis.

The visible spectrum of the molybdoferredoxin is a broad absorption between 300-600 nm (45). Upon oxygen exposure of the Klebsiella pneumoniae and Azotobacter vinelandii enzymes, the spectrum undergoes a slight increase in absorbance over the same region (45). Similar exposure of the Clostridium pasteurianum molybdoferredoxin resulted in the appearance of a new peak at 435 nm (45). The spectrum of azoferredoxin is a broad absorption between 400-650 nm (45).

In the same study by Shah and Brill (45), EPR studies were done with emphasis on the effects of oxygen exposure. Oxygen exposure of molybdoferredoxin resulted in "slight" diminution of signal strength and the generation of a strong signal at g = 2. Similar treatment of the azoferredoxin resulted in complete loss of signal.

In addition to oxygen lability, azoferredoxin was found to be cold labile. Storage at 0-4°C for 96h resulted in a 39 percent loss of activity (45).

Recent data indicate that, besides its role in the catalysis of dinitrogen reduction, molybdenum may also have a regulatory function in the synthesis of nitrogenase components. In a study by Brill et al (10) it was demonstrated that cells of Klebsiella pneumoniae, derepressed in a molybdenum-free medium, synthesized neither active component I nor II. A similar effect was noted when K. pneumoniae cells were derepressed in a medium containing both molybdenum and tungsten. With the exception of the Rhodospirillum rubrum nitrogenase system, tungsten has been found to be a potent, competitive inhibitor of the molybdenum-mediated catalysis in nitrogenase systems (10,37). Kinetic data derived from this study indicated that molybdenum is an obligate requirement for both the synthesis and the function of nitrogenase systems (10). Such data, coupled with component titration also lend credence to the co-transcription of the nif genes (10). With regard to the metal cofactors

of molybdoferredoxin, vanadium has been shown to replace molybdenum, yielding active nitrogenase (10,36,37). When cells of Azotobacter vinelandii are derepressed in media containing tungstate, an inactive molybdoferredoxin is produced (10). The inactivation by tungstate may be alleviated in vivo but not in vitro by the subsequent addition of molybdate (10).

As stated above, azoferredoxin is the electron carrier to the active site of molybdoferredoxin. In Azotobacter spp. axoferredoxin can be reduced by either of two electron carriers. The first is ferredoxin of molecular weight 14.5 Kd having eight -(FeS)- units (4,27). The other is azotoflavin, a flavodoxin, having a molecular weight of 31 Kd and utilizing a dissociable flavin mononucleotide prosthetic group (4,54). In the past, it was thought that ferredoxin and flavodoxin functioned sequentially, mediated by a coupling factor (54). It is now currently held that both azotoflavin and ferredoxin can directly reduce azoferredoxin (4,54).

In addition to its function in the reduction of molecular nitrogen, nitrogenase also reduces protons forming molecular hydrogen (54). This side reaction, termed the ATP-dependent hydrogenase function, is competitive with nitrogen reduction. The ATP-dependent hydrogenase reaction is, essentially, irreversible (42). Given the cellular expenditure of energy, it is difficult to attach an advantage

to this function although the ATP-dependent hydrogenase is common to all known nitrogenase systems (42,54).

The reduction of molecular nitrogen to ammonia is a thermodynamically favorable reaction, but places a great energy demand on biological systems. Current measurements indicate the hydrolysis of two ATP's per electron transfer. This yields a theoretical ATP demand of 12 ATP per nitrogen molecule reduced. However, the best estimates of ATP demand are in the range 15-24 ATP per nitrogen molecule reduced (9). It is likely that this rather high figure also represents competition between protons and dinitrogen for available electrons.

Besides the reduction of protons and molecular nitrogen,. nitrogenase is also capable of reducing the triple bonds of a wide variety of "artificial" substrates. Among the most widely used artificial substrates are nitriles, acetylene, cyanide, azides, and nitrous oxide which are reduced to alkanes + ammonia, ethylene, ammonia + methane, ammonia + nitrogen, and nitrogen + water respectively (42). Of these, acetylene represents the most widely utilized substrate for the quantitation of biological nitrogen fixation (15,27, 44,50,53). The product of the reaction, ethylene, may be resolved and quantitated by gas chromatographic techniques. The acetylene reduction assay may be performed <u>in vivo</u>, <u>in vitro</u>, or <u>in situ</u> (15, 27,44,45,50,53). For the <u>in vitro</u> assay, crude or purified extracts may be assayed under anaerobic conditions. In such systems,

dithionite serves as reductant and substrate quantities of magnesium ion and ATP are also included (42,45). The optimal Mg:ATP ratio has been determined to be 1:2 (42). Due to the fact that acetylene reduction is a two electron event and nitrogen reduction is a six electron event, and as little as .2 atm. acetylene inhibits the ATP-dependent hydrogenase function, direct estimation of the nitrogen fixing capacity of a sample is not possible without correction (31,42, 45). The best estimate of the acetylene:nitrogen efficiency ratio is 3.86:1 (45).

Ammonia Assimilation

Ammonia is the central most important nitrogen source in the microbial world. Ammonia is preferentially utilized as a nitrogen source even in the presence of nitrate or molecular nitrogen (12,15, 25,26,52). In order to be of physiological utility, ammonia must be linked to a carbon-containing species. The biosynthetic processes through which this union occurs are termed ammonia assimilatory mechanisms.

Among the majority of the prokaryotes and lower fungi, ammonia assimilation proceeds via the synthesis of glutamine, glutamic acid, carbamyl phosphate, and alanine (5,12,15,25,26). The syntheses of

these compounds are respectively mediated by glutamine synthetase, glutamate dehydrogenase or glutamate synthase, carbamyl phosphate synthetase, and alanine dehydrogenase (12).

Glutamine Synthetase

Glutamine synthetase (L-glutamate:ammonia ligase ADP forming) and its accessory regulatory system comprise one of the most intricate enzyme systems known in the microbial world (3,48). The enzyme catalyzes the irreversible synthesis of glutamine from ammonia and glutamic acid. ATP is required as an energy source in the reaction along with magnesium ion (1,3,48). The glutamine synthetase of Gram negative bacteria exists as a dodecameric protein having identical subunits whose molecular weights are 50-55 Kd (29,46,48). In purified preparations, glutamine synthetase tends to form tubular aggregates depending on the level of divalent metal cations present in the preparation (1,3,29,46,48).

The glutamine synthetase of the Enterobacteriaceae and the Azotobacteraceae are known to be regulated by, at least, six different mechanisms (1,25,29,39,48). These mechanisms are: (1) covalent modification via adenylylation and deadenylylation, (2) concerted feedback inhibition by several end-products of glutamine metabolsim,

(3) equilibrium between taut (active) and relaxed (inactive) forms under the influence of divalent metal cation concentrations, (4) modulation of catalytic activity via the differing divalent metal cation specificities of adenylylated versus deadenylylated forms of the enzyme, (5) repression and derepression of enzyme synthesis in response to intracellular levels of glutamine pools, (6) feedback inhibition by nucleoside triphosphates.

The regulation of glutamine synthetase activity by reversible adenylylation/deadenylylation cycles appears to be ubiquitous among Gram negative bacteria (1,3,4,7,9,12,25,29,38,48,54). There is also evidence of this mode of regulation among the cyanobacteria as well (1,3,12,29,48). Under conditions of metabolic nitrogen excess, the enzyme is covalently modified via adenylylation. There is one adenylylation site per subunit and the linkage site for the AMP moiety (derived from ATP) is a single tyrosine residue (1,3,38,46, 48).

Under the conditions of nitrogen limitation, the adenylylated, less active glutamine synthetase, can be reactivated by phosphorylytic cleavage of the (tyr-O-AMP) linkage (1,3,33,38,46,48).

The adenylylation of glutamine synthetase has several effects on the biosynthetic activity of the enzyme. Glutamine synthetase in the deadenylylated form is dependent on magnesium ions for both the biosynthetic and glutamyl-transferase activities (46). Adenylylated,

and therefore less active, glutamine synthetase is dependent on manganese ions for its biosynthetic and transferase activities (1,3, 38,48). Adenylylation also increases the enzyme's Km for ammonia (38). The pH optimum for the biosynthetic reaction shifts from 8.0 to 6.9 upon adenylylation (1,38,48). Both the biosynthetic and transferase activities become far more susceptible to feedback inhibition upon adenylylation of the enzyme (38). Examples of feedback inhibitors are tryptophan, alanine, glycine, AMP, glucosamine-6-phosphate, CTP, carbamyl phosphate, and histidine (38,46,48). There is evidence of multiple, allosteric binding sites on the enzyme for these inhibitors and the variety of allosteric effectors tends to reflect the, rather, central positions of glutamine in microbial nitrogen metabolism (38).

Both adenylylation and deadenylylation of glutamine synthetase are catalyzed and regulated by a complex "sensor-effector" system known as the regulatory cascade (1,3,38,39,46,48). The regulatory cascade consists of four enzyme activities (1,3,38,39,46,48). The system was initially detected in Escherichia coli.

A bifunctional adenylyltransferase (ATase) catalyzes the interconversion of glutamine synthetase between the adenylylated and deadenylylated forms (38,46). ATase of E. coli has a molecular weight of 120-140 Kd (38,46).

The P-II regulatory protein modulates the mode of ATase activity (38). E. coli and Pseudomonas putida P-II have molecular weights of 44 Kd and exist as tetramers of apparently identical subunit types (1). P-II, in a manner analogous to glutamine synthetase, may exist in two, interconvertible forms. When P-II is unmodified it shifts the mode of ATase activity toward adenylylation and is referred to as P-IIA (1,38). When P-IIA is covalently modified via uridylylation it shifts the operational mode of ATase toward deadenylylation and is referred to as P-IID or P-II*(UMP) (1,3,38,39,46,48). The uridylylation site of P-II has been shown to be a single tyrosine residue.

The UR-UTase complex consists of two, inseparable activities (1,38,48). The UTase, uridylyltransferase, activity catalyzes the uridylylation of P-II utilizing UTP (1,38,48). The UR, uridylyl-removing, activity catalyzes the deuridylyation of P-IID (1,38,48). Efforts to separate these two activities have, thus far, been unsuccessful (1). The two activities tend to co-purify with all known purification techniques (1,38,48). The UR-UTase complex comprises the "sensor" portion of the cascade in that its catalytic mode is subject to control by the balance between glutamine:2-oxoglutarate and Mg:Mn. High levels of glutamine signal an over-supply of available nitrogen and the UR-UTase complex "responds" by switching to its UR mode (1,38,48). 2-Oxoglutarate acts as a negative

effector of the UR function (38). In addition, UR requires ATP and 2-oxoglutarate in the presence of high magnesium ion concentrations, but no additional cofactors in the presence of high manganese ion concentrations (48). A shift in the glutamine:2-oxoglutarate balance toward 2-oxoglutarate signals nitrogen limitation. UR-UTase responds, accordingly, by shifting its catalytic mode to uridylylation (UTase) (48). This activity requires ATP and magnesium or manganese ions in addition to 2-oxoglutarate as cofactors (1,38,48). Glutamine and inorganic phosphate are negative effectors of UTase-mediated catalysis (1,3,38,46,48).

To date, the regulation of glutamine synthetase activity by covalent modification has not been detected among Gram positive bacteria (1,26,38,48). The mode of glutamine synthetase regulation in these organisms appears to be under an entirely different control mechanism(s).

In addition to the controls exerted on glutamine synthetase, there is a growing body of evidence which indicates that, in some bacterial systems, glutamine synthetase may exert regulatory control over other enzymes involved in nitrogen metabolism (6,7,20,26,33,38). Recent evidence (1974) has suggested a role for glutamine synthetase in the regulation of nitrogenase (8,20). Methionine sulfoximine, an analogue of glutamine, is a potent inhibitor of glutamine synthetase activity and a concentration of 0.01 mM was found to result in 70%

inhibition of the enzyme (8). Gordon and Brill (20) discovered that the addition of methionine sulfoximine to cultures of K. pneumoniae and A. vinelandii resulted in derepression of nitrogenase synthesis in the presence of excess ammonia. Another reference to the same work (9) indicated that addition of methionine sulfoximine to cultures of K. pneumoniae resulted in both the derepression of nitrogenase synthesis and the excretion of ammonia into the surrounding medium. Methionine sulfoximine binds irreversibly to the active site of glutamine synthetase "locking" it into the active conformation and preventing adenylylation (6,8). Similar results were obtained for glutamine synthetase constituitive mutants of K. pneumoniae (8). The currently accepted hypothesis is that unadenylylated enzyme acts as a positive effector in the transcription of nif cistron(s) (6,20). This, however, does not preclude the possibility of other mechanisms.

Glutamate Synthase

Glutamate synthase [glutamine (amide):2-oxoglutarate NADP amido transferase oxido-reductase] catalyzes the synthesis of glutamate from glutamine and 2-oxoglutarate. The stoichiometry of the reaction is such that two equivalents of glutamate are derived per glutamine. Glutamate synthase is also referred to in the literature

as GOGAT (7,34). Glutamate synthase exists as an octameric protein of two, dissimilar subunit types having molecular weights of 135 and 53 Kd in Klebsiella aerogenes and possesses 32 Fe and 32 labile S (7,34,38).

Tempest et al discovered that extracts of Aerobacter (Enterobacter) aerogenes, when grown under conditions of nitrogen limitation, possessed an NADP/NADPH-dependent pathway for the synthesis of glutamate. This activity was not due to glutamate dehydrogenase, as there was no demonstrable activity for this enzyme under the conditions of nitrogen limitation (7,34,51).

Among the bacteria and cyanobacteria there exist, at least, two major ammonia assimilatory pathways (7,12). Under conditions of ammonia oversupply, glutamate dehydrogenase serves as the major assimilatory enzyme. Glutamate dehydrogenases in bacterial systems have relatively high Km's for ammonia, ranging between 12-300 mM (7,12,34). Thus, under conditions of ammonia limitation glutamate dehydrogenase is incapable of an efficient rate of glutamate synthesis. Ammonia limitation leads to the derepression of glutamine synthetase and glutamate synthase, while glutamate dehydrogenase is either repressed or only marginally functional (7,12,34).

Glutamine synthetase and glutamate synthase act in concert yielding the net synthesis of glutamate from ammonia, ATP, and 2-oxoglutarate (3,7,12,25,48). This enzyme pair constitutes the low

Km or high affinity ammonia assimilatory pathway of the majority of prokaryotes (1,3,7,12,25,48). Glutamate dehydrogenase represents the high Km or low affinity ammonia assimilatory pathway (7).

With regard to the "switchover" between the two ammonia assimilatory systems, current evidence suggests that active, deadenylylated glutamine synthetase acts as a repressor of glutamate dehydrogenase (34). It has also been suggested that active glutamine synthetase acts as an inducer of glutamate synthase in bacterial systems in which the synthesis of glutamate synthase is not constitutive (34). Metabolic events which lead to the inhibition and repression of glutamine synthetase relieve the repression of glutamate dehydrogenase (1,3,7,12,25,48).

Glutamate Dehydrogenase

Glutamate dehydrogenase catalyzes the synthesis of glutamate from 2-oxoglutarate and ammonia or glutamine (12,19). Depending on the source, the enzyme utilizes either NAD/NADH or NADP/NADPH as a cofactor (7,12,19,38). The enzyme, typically, exists as a hexameric protein of molecular weight 360-366 Kd composed of identical subunit types, in bacterial systems (12,19,38).

With regard to ammonia assimilation, glutamate dehydrogenase primarily fulfills a catabolic role (5,12,33). However, a few bacteria, cyanobacteria, and lower fungi have glutamate dehydrogenases which serve in an ammonia assimilatory capacity (5,12,33). Among the lower fungi, Neurospora, Candida, and Saccharomyces there exist two, distinct glutamate dehydrogenases (12,19). One form of the enzyme is similar to that which is found in bacteria. It is NAD/NADH-linked, possesses a high Km for ammonia, and is relatively inactive at limiting levels of ammonia (12,19,33). The other form of the enzyme is NADP/NADPH-linked and is the major ammonia assimilatory enzyme in these organisms (12). The latter form of the enzyme has a relatively low Km for ammonia and is repressed by excessive environmental levels of ammonia (12,19).

With the exception of a few cyanobacteria (eg. Anabaena cylindrica), organisms having dual glutamate dehydrogenases do not depend on glutamine synthetase/glutamate synthase as the primary ammonia assimilatory system (12).

In the alga Chlorella pyrenoidosa, a single form of glutamate dehydrogenase serves a dual function (12). The enzyme is NADP/NADPH-linked and possesses a Km for ammonia of 0.5 mM (12).

Carbamyl Phosphate Synthetase

Carbamyl phosphate synthetase catalyzes the synthesis of carbamyl phosphate from bicarbonate ion and either ammonia or glutamine. The reaction consumes two ATP and N-acetyl glutamate is required as a cofactor (12,18,24,30). Carbamyl phosphate is a high energy, unstable coprecursor in the synthesis of arginine, proline, and pyrimidine nucleosides (30).

The role of carbamyl phosphate synthetase in microbial ammonia assimilation is dubious given the following observations. In E. coli, for example, the enzyme exhibits a higher affinity for glutamine than ammonia (12). The Km of the E. coli enzyme for ammonia is 93 mM (12). This is in marked contrast to the hepatic enzyme of mammals, which exhibits a higher affinity for ammonia than glutamine (12,18,24,30). The E. coli enzyme is also subject to severe inhibition by the end-products of carbamate metabolism (12).

Alanine Dehydrogenase

Alanine dehydrogenase catalyzes the amination/deamination of alanine, with pyridine nucleotides as cofactors (12,40).

Among bacteria, alanine dehydrogenase is now known to serve a catabolic rather than biosynthetic role in nitrogen metabolism (12, 40). As recently as 1974, alanine dehydrogenase was thought to serve a major ammonia assimilatory role in the cyanobacterium, Anabaena cylindrica (12). More recent evidence has indicated a catabolic role of the enzyme in A. cylindrica as well (40). One of the factors which lead to the former misconception is the constituitive nature of the enzyme in this organism (40). It is now accepted that in most prokaryotes the glutamine synthetase/glutamate synthase pathway constitutes the major ammonia assimilatory pathway under conditions of nitrogen limitation (18,24,25,26,28,29,33,34,38,40). In Anabaena cultures provided with excess ammonia, the alanine dehydrogenase pathway may serve an efficient assimilatory function (40).

Alanine dehydrogenase, in Anabaena spp., exists as a hexameric protein of identical subunits with a total molecular weight of 270 Kd (40). The enzyme is NAD/NADH-linked and has low activity with NADP/NADPH (40). The Anabaena enzyme is active in both amination of pyruvate and deamination of alanine and has slight activity (Km 3 mM) with oxalacetate (40). It is active in both vegetative cells and heterocysts but has a higher level of activity in nitrogen-starved cultures than those fixing nitrogen (40).

Ammonium Transport Systems of N_2 Fixing Bacteria

Clostridium pasteurianum is an obligately anaerobic, free-living, nitrogen fixing bacterium. Kleiner (26), in a comprehensive study of the regulation of ammonium uptake in this organism, has determined that the major ammonia assimilatory pathway involves the glutamine synthetase/glutamate synthase pair. The glutamine synthetase of this organism, in contrast to that of Gram negative bacteria, is not regulated by adenylylation/deadenylylation cycles (26). Upon the addition of ammonium ion, no shift was found in the levels of activity of the aforementioned enzymes (26).

Studies of ammonia-induced effects on amino acid pools were supportive of the hypothesis that glutamine synthesis is the major ammonia assimilatory mechanism in this organism. Kleiner (26) states that ". . .the increase in nitrogen influx seemed to propagate in a wavelike manner through the different metabolic pathways and reached the pools of the different amino acids after various times . . ." In Kleiner's study, only the glutamine pool responded to increased ammonia influx by significant and immediate increase (26).

Ninety percent of added ammonium ion is absorbed immediately by this organism and this transport is independent of the anion of the ammonium salt employed (26).

Ammonium ion uptake and assimilation are far more complex in A. vinelandii. Kleiner (25), in a study similar to the one outlined above, elucidated several ammonium ion transport mechanisms in this organism. The glutamine synthetase in this organism resembles that of E. coli in that it is also regulated by adenylylation/deadenylylation cycles (25,29).

Under conditions of ammonia limitation or nitrogen fixation, glutamine synthetase and glutamate synthase serve as the ammonia assimilatory system in A. vinelandii (25). When ammonium ion levels are increased to the point of oversupply, the above system is inhibited and repressed and glutamate dehydrogenase becomes the major assimilatory enzyme (25).

Unlike the clostridial system, ammonium transport in A. vinelandii is highly dependent on the nature of the anion of the ammonium salt employed (25,26). Upon addition of ammonium citrate, lactate, or acetate, ammonium uptake is rapid and immediate. However, if sulfate, chloride, or phosphate salts are employed acid production is concomitant with, a slower, ammonium ion uptake (25). Acid production is thought to be due to the uptake and reexcretion of the $SO_4^{=}$, Cl^{-}, or $PO_4^{\equiv}$ resulting in the formation of the conjugate acid of the salt employed (25). The more efficient uptake of acetate, citrate, and lactate salts has been attributed to a symport mechanism in which these anions are more easily transported than chloride,

sulfate, or phosphate (25). This anion selectivity was observed to halt after 2h incubation. After this lag, residual ammonium ion was rapidly taken up in a manner independent of the anion (25). This strongly indicated that A. vinelandii had, at least, two ammonium ion translocases; one of which was inducible (25). Another property of the A. vinelandii ammonium translocase system is its obligate dependence on oxygen (25). Kleiner (25) hesitates to attribute this finding to an ATP-dependent transport mechanism since experimental evidence does not reveal the cessation of ATP-dependent metabolism as a result of transient anaerobiosis. However, A. vinelandii has been found to maintain a 100-fold ammonium ion gradient across its plasma membrane under conditions of nitrogen fixation (25). It seems likely, given this fact, that ammonium transport is energy linked.

A recent study has demonstrated that thirty percent of the glutamine synthetase of A. vinelandii is irreversibly bound to the plasma membrane (29). Glutamine synthetase as an ammonium ion translocase is an attractive model since it parallels the role of glutamine synthetase in mammalian systems (11,25,29).

Ammonium uptake in Klebsiella pneumoniae is, somewhat similar to that of A. vinelandii (25,28). Upon the addition of slight excess of ammonium ion, there is rapid and immediate uptake on the order of fifty percent (28). After a ninety minute lag, a new transport system (late translocase) becomes operative and the remaining

ammonium ion is absorbed (28). In marked contrast to the A. vinelandii system, K. pneumoniae transport of ammonium ion is independent of the anion of the ammonium salt employed (28). Further, citrate is not absorbed when supplied as ammonium citrate; as it is in A. vinelandii (25,28). Kleiner (28) attributes this to the uptake of ammonia rather than ammonium ion.

Effects of Ammonium Ion Addition on Nitrogen Fixation

The elevation of ammonium ion concentration in cultures of nitrogen-fixing bacteria results in a rapid decline of nitrogenase activity (15,25,26,28,43,50,52). This decline of activity is due either to repression and/or inhibition of nitrogenase. There is, however, highly convincing evidence that ammonium ion is not the agent responsible for this decline of nitrogenase activity (6,8,20,25,26,28, 43,52).

When C. pasteurianum is shifted from N_2 growth to growth on ammonia (ammonium ion) there is an immediate decline of nitrogenase activity (26). The influx of ammonium ion has no effect on the activities of glutamine synthetase, glutamate dehydrogenase, or asparagine synthetase (26).

Of the amino acid pools detectable in C. pasteurianum, only the glutamine pool significantly expands upon addition of ammonium ion (26). The rate of glutamine pool expansion has been determined to be inversely correlated with the level of nitrogenase activity (26). Intracellular ammonia pools remain at a constant level, under the above conditions (26). This behavior lends credence to the hypothesis that ammonia is, only, indirectly related to nitrogenase regulation. Kleiner (26) concludes that nitrogenase regulation in C. pasteurianum is due to a repression mechanism rather than direct inhibition.

Nitrogenase activity in A. vinelandii also declines rapidly upon elevation of environmental ammonium ion concentration (25). Unlike the clostridial system, glutamine synthetase activity shows a slight increase in activity when growth is shifted from N_2 to NH_4^+ growth (25,26). Pretreatment with chloramphenicol does not inhibit the elevation of glutamine synthetase activity (25). It, therefore, seems that the increase in glutamine synthetase activity is due to deadenylylation rather than de novo biosynthesis (25). The transient increase in glutamine synthetase activity may serve to maintain intracellular ammonia pools within functional norms (25). Supporting evidence for this hypothesis is given by the fact that intracellular ammonia and the 100-fold trans-membrane ammonia gradient is maintained

(25). There is also evidence of glutamine synthetase involvement in the induction of the late ammonia permeases (25).

The activity of K. pneumoniae nitrogenase also declines upon addition of excess ammonium ion (28). However, the initial decline in activity is not as steep as that observed in A. vinelandii (25,28). In chemostatic cultures of K. pneumoniae, the addition of ammonia results in an exponential decline of nitrogenase activity. This is best explained as repression followed by "wash-out" kinetics (21,28). In a study by Kleiner (28) the level of nitrogenase was observed to "closely parallel" that state of adenylylation of glutamine synthetase. This presents additional evidence of the possible involvement of glutamine synthetase in the regulation of nitrogenase.

When nitrogen fixing bacteria, grown on ammonia, deplete their nitrogen supply a growth lag of 60-90 min. occurs. Subsequent to this lag, growth resumes supported by nitrogen fixation (15,43,50,52). This diauxic growth phenomenon is common among many nitrogen fixing bacteria undergoing nitrogenase derepression after growth on ammonia (15,43,50,52).

The duration of the diauxic lag phase is not absolute. In C. pasteurianum, for example, the duration of the lag is partially dependent on the number of generations the cells were grown on ammonia (15). The lag phase may also be shortened by the addition of amino acids and yeast extract (15,55). This, presumably, prevents

nitrogen starvation and permits the de novo biosynthesis of nitrogenase components without dependence on protein turnover for amino acids.

Seto and Mortenson (43) have performed a detailed study on the timing of events which take place during the diauxic lag phase of C. pasteurianum. Transcription of nitrogenase mRNA's is initiated 25-30 min. into the lag. Complete transcription of nitrogenase mRNA's requires 6-8 min. after initiation. These mRNA's are unstable, having a half life of 1-2 min. Evidence exists that nitrogenase regulation, mediated by ammonia, occurs at the level of mRNA synthesis and/or turnover (43,52,55). Active components I and II are completely synthesized 45 min. into the lag. Presumably, 15-45 additional minutes are required before nitrogen fixation begins to support growth.

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THE REGULATION OF NITROGEN FIXATION IN *AZOTOBACTER VINELANDII*: A BIOLOGICAL SYSTEMS APPROACH

Introduction

The precise mechanism(s) through which nitrogenase activity is regulated is not precisely understood. The addition of ammonia (ammonium ion) to nitrogen fixing cultures of *A. vinelandii* results in the rapid decline of nitrogenase activity (4). However, the direct involvement of ammonia in nitrogenase inhibition and/or repression has been refuted (4). The addition of ammonia must, therefore, initiate some sequence of regulatory events which result in the inhibition/repression of nitrogenase activity. Within this sequence of regulatory events there must be a sensory mechanism which detects the condition of ammonia oversupply. Given the energy demands of nitrogen fixation, it would be advantageous for the organism to cease fixation of nitrogen whenever biological nitrogen demand is met by alternative sources. The studies made by Gordon and Brill (2) indicate that the ammonia assimilatory system is involved in some phase of the regulation of nitrogenase activity under the conditions of ammonia excess.

It is likely that the event which signals ammonia oversupply is embedded within the matrix of the assimilatory system.

Under conditions of nitrogen limitation, the rate of ammonia synthesis may be a rate limiting step in nitrogen metabolism and growth. This would imply that, under steady-state conditions, the products of ammonia assimilation are being utilized at their maximum rates. The addition of exogenous ammonia would over-ride the rate limit imposed by nitrogenase catalysis. There are, however, limits on the rate at which the products of ammonia assimilation can be utilized. The addition of ammonia to a level which exceeds the maximum level necessary for optimal growth may promote the accumulation of the initial products of ammonia assimilation, glutamine and glutamate. The accumulation of glutamine, for example, may be the signalling event sensed by the system which regulates nitrogenase activity. There are, however, a number of nitrogenous components within the system which may also serve in this capacity. An a priori approach toward an understanding of nitrogenase regulation would not be feasable, as the number of possible component combinations approaches astronomical proportions. If the dynamics of the system's responses to ammonia excess were better characterized, then it might become possible to delineate some of the parameters associated with nitrogenase regulation. Understanding the behavior of the system would permit intelligent selection

and detailed study of those response parameters which correlate significantly with the dynamics of nitrogenase activity.

The focus of this study is the preliminary parameterization of the dynamic responses of nitrogenase, the ammonia assimilatory system, and ammonia utilization efficiency to the addition of excessive levels of ammonia. When possible, regression and interpolation methods were utilized to derive algebraic expressions for the observed responses. These expressions may be useful in a subsequent and more detailed study of nitrogenase regulation. One must bear in mind, however, that valid predictions and/or applications based on these expressions may only be made under the conditions for which they were derived.

Materials and Methods

Azotobacter vinelandii ATCC 12837 was employed throughout this study. For batch culture, cells were grown in Burk's buffer with one percent glucose (8). For chemostatic culture, cells were grown in a modified Burk's buffer formulated by Kleiner (4). In both cases incubation was at 30°C. When employed, ammonium salts were sterilized by filtration and added aseptically.

Acetylene Reduction Assay--The *in vivo* assay for acetylene reduction was employed throughout this study. Active nitrogenase reduces acetylene to ethylene, which can be detected and quantitated via gas chromatographic techniques. For the assay, 2 cc of culture were added to 12 cc serum vials fitted with rubber septa. Acetylene (2 cc) was added to the sealed vials with a syringe after the withdrawal of an equivalent volume of the vial vapor phase. The samples were then incubated in a horizontal position on a platform, rotary water bath at 30°C with vigorous agitation. A Varian model 1440 gas chromatograph, fitted with a 6' Porapak N column and a hydrogen flame ionization detector, was employed for the quantitation of ethylene production.

Glutamine Synthetase--The whole cell, biosynthetic assay of Bender *et al* (1) was utilized for the estimation of glutamine synthetase activity. In the assay, active, unadenylylated enzyme catalyzes the condensation of glutamate and hydroxylamine to form glutamylhydroxamate. The addition of acidic ferric chloride results in the formation of a colored complex which has an absorbance at 540 nm and can be quantitated spectrophotometrically. For the assay, cells were harvested from culture via centrifugation at 4°C. The pellet was resuspended in a buffer consisting of imidazole-HCl (94 mM @ pH 7.5), $MgCl_2$ (56 mM), and CTAB (94ug/ml). CTAB

(cetyltrimethylammonium bromide) renders the cell membrane permeable to the reactants in the assay mixture. Samples of the suspension were used in the assay. The reaction was initiated by the addition of ATP and halted by the addition of the acidic ferric chloride stop mixture (1). Units of enzyme activity were recorded as micromoles glutamyl-hydroxamate formed per minute per unit volume of culture.

Glutamate Dehydrogenase--Glutamate dehydrogenase was assayed by the colorimetric assay of King (3). Cells were prepared for the assay by sonic disruption in 0.07M phosphate buffer, pH 7.0. The assay quantitates the biosynthetic activity of the enzyme by measuring the quantity of 2-oxoglutarate consumed during the reaction, the remainder of which is detected by reaction with 2,4-dinitrophenyl-hydrazine. The dinitrophenylhydrazones formed absorb in the 385-400 nm region when exposed to alkaline conditions. Units of enzyme were recorded as nanomoles 2-oxoglutarate consumed per minute per unit volume of culture.

Cyclic AMP Radioimmunoassay--The quantitation of 3',5' cyclic adenosine monophosphate was performed according to the protocol included in the cAMP RIA kit supplied by New England Nuclear Corp., which was first developed by Steiner et al (7). The technique involves the competition for antibody binding sites (dynamic)

between cAMP in the sample and I^{125}-labelled cAMP. The residual, bound I^{125} cAMP was quantitated in a Packard Tricarb Gamma Counter. Cells from batch cultures were isolated on membrane filters. The filters were then plunged, immediately, into 13 X 100 mm tubes containing 0.1N HCl. The tubes were heated at 95°C for 20 min. to extract soluble material. Debris and filters were removed via centrifugation and the supernatant fluid was decanted and evaporated under a stream of dry N_2 to remove the HCl. The dried residue was then resuspended in water and aliquots were removed from the solution for the assay. Cells from chemostatic cultures were added directly to 13 X 100 mm tubes containing concentrated HCl such that the final HCl concentration was 0.1N. After this step, samples were prepared in the manner outlined for batch cells beginning with the heat treatment step. Samples for the assay were acetylated with acetic anhydride, according to the protocol supplied with the kit, to increase the sensitivity of the assay.

Ammonia--Quantitation of ammonium ion concentration was made via the use of Nessler's reagent. For the assay, samples were centrifuged to remove cellular material and the supernatant fluid was employed in the assay. Ammonia reacts with the reagent to form a colored complex which can be measured spectrophotometrically at 490 nm.

Chemostat--The chemostat vessel consisted of a 1 liter high-from beaker with a glass overflow port attached. The vessel was mounted on a brass tripod assembly and covered with a teflon-lined brass cover plate. Addition of medium and inoculum were facilitated via stainless steel tubes which were welded through the cover plate. Constant temperature was maintained by placing the assembly in a thermostatted water bath. When utilized, ammonia was supplied in the form of ammonium acetate. The dilution rate, D, was 0.26/hr and the air flow rate was 6-8 liters per minute. The working volume was 475 ml. For quick-pulse studies, the initial ammonium concentration was 10 mM (Appendix A). For slow-pulse studies, the reservoir ammonium ion concentration was 10 mM (Appendix A).

RESULTS AND CONCLUSIONS

Dependence of Growth Rate on Nitrogen Availability

A test of the hypothesis that nitrogen fixation is a growth rate limiting step in A. vinelandii was an initial focus of this study. If the hypothesis is correct, then the addition of ammonia (ammonium ion) to nitrogen fixing cultures should result in shorter generation times as a rate limiting step will have been bypassed.

Contrary to expectation, growth rates did not significantly increase when cultures were supplemented with ammonia at concentrations less than or equal to 2 mM. Generation times were actually increased when ammonia concentrations less than 2 mM were employed and did not significantly decrease until a concentration of 10 mM ammonia was supplied. This latter result is, however, supportive of the hypothesis, and provides a measure of the threshold level of ammonia needed for an efficient bypass of the rate limit imposed by nitrogenase catalysis (i.e. ammonia utilization efficiency) (Figures 1 and 2). However, the growth rate data at lower concentrations appeared somewhat anomalous. Given the hypothesis that the rate of

Figure 1.--Overall representation of ammonia dependent growth rate study.

(■) control point (●) trial 1
(▲) trial 2

A series of flasks having the recorded ammonium ion concentrations were inoculated and growth rate measured turbidimetrically.

Figure 1

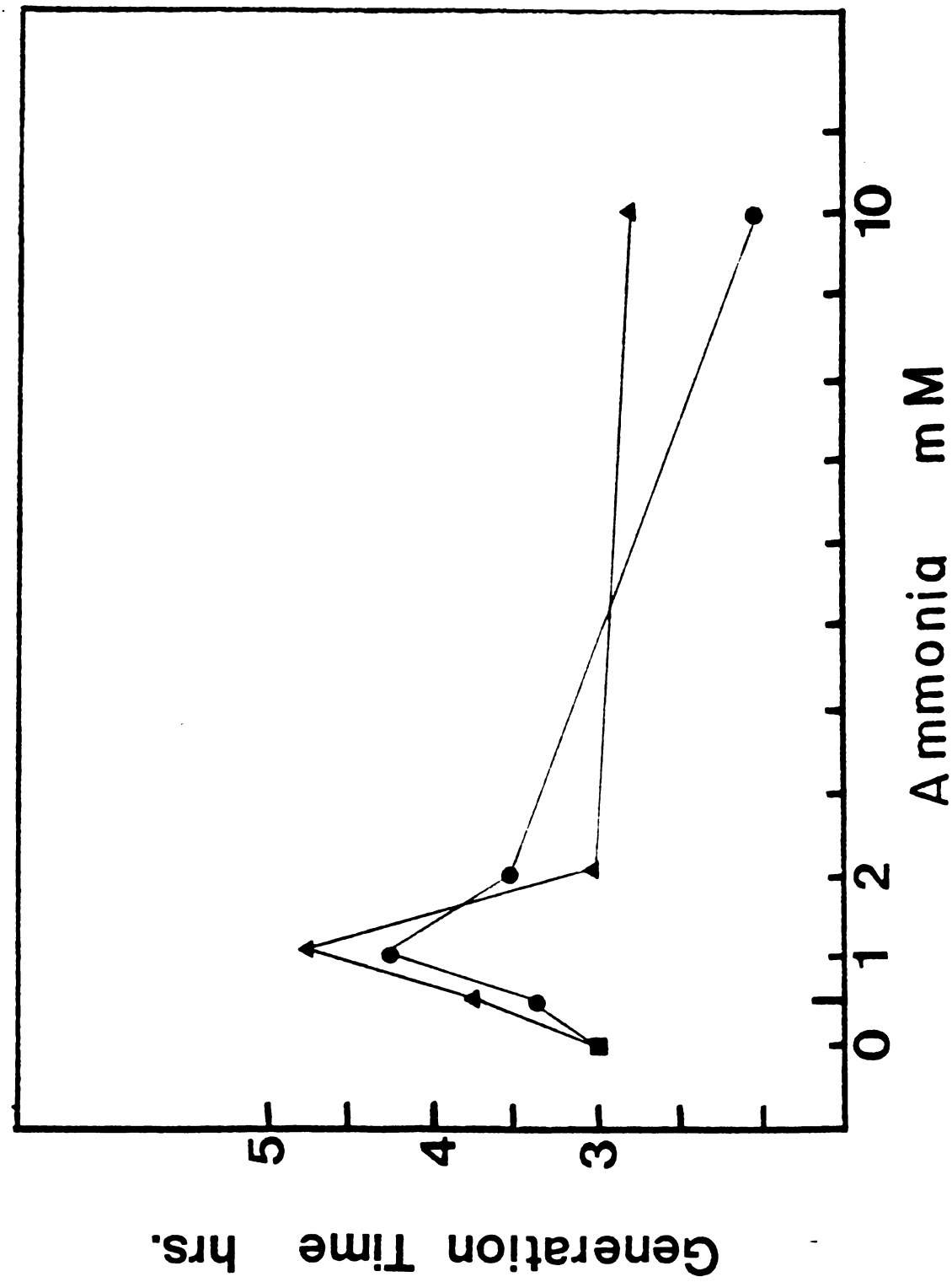


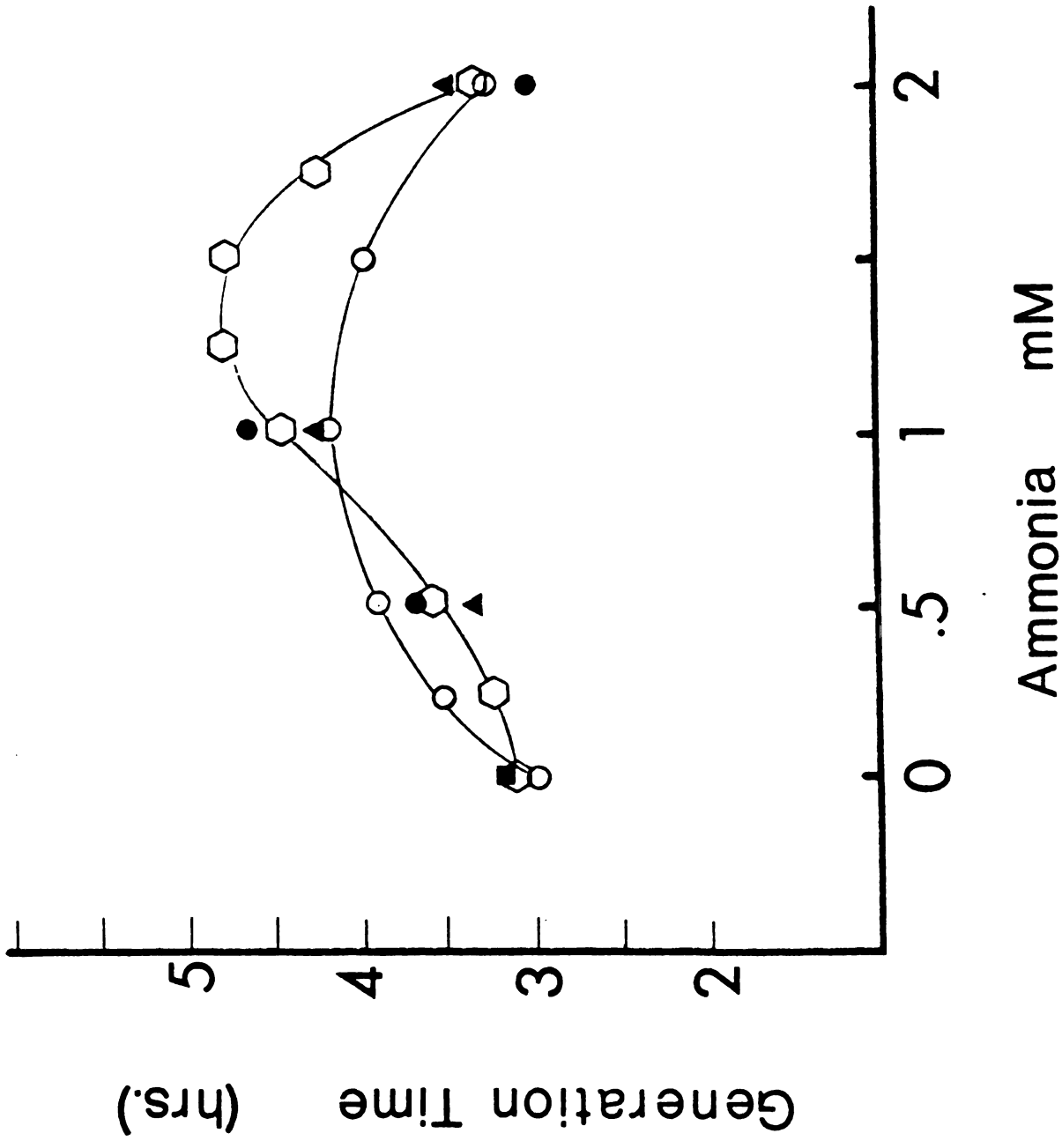
Figure 2.--Enlargement of the latch region or region of anomalous growth rates (0-2 mM NH_4^+).

(▲) trial 1 (●) trial 2

(○) P(2) (⬡) P(3)

P(2) and P(3) denote quadratic and cubic least-squares regression curves derived from the data. See text and Appendix B for a more thorough explanation.

Figure 2



nitrogen fixation imposes an overall rate limit on metabolism, it was expected that NH_4 supplementation would enhance growth rates. The data indicate that a 2 mM ammonium ion concentration is barely sufficient for the maintenance of growth at the rate observed under conditions of nitrogen fixation (Figures 1 and 2). There was no detectable nitrogenase activity in cultures supplemented with an ammonium ion concentration above 1 mM. Given these data, the hypothesis was formulated that the growth rate behavior observed at ammonia concentrations less than 2 mM was due to an ammonia-induced shift from a high affinity ammonia assimilatory system to one of lower affinity. Data obtained by Kleiner (4) indicate that glutamine synthetase and glutamate synthase comprise the former system and glutamate dehydrogenase the latter. The relationship between the two systems was envisioned as a "swinging latch" which channels ammonium ions through one of the two pathways; depending on the level of exogenous ammonia. To test this "latch" hypothesis N_2-fixing cultures were pulsed with 2 mM ammonium ion and assayed for glutamate dehydrogenase activity at various times thereafter. The activity of glutamate dehydrogenase does, indeed, increase upon the addition of ammonia (Figure 3). This datum was viewed as supportive of the latch hypothesis. The difference in Km's for ammonia of the two uptake systems may explain the growth rate decrease at ammonia concentrations less than 2 mM, as the actual uptake of ammonia is less efficient. Thus, ammonia dependent

Figure 3.--Glutamate Dehydrogenase activity response to 2 mM NH_4 pulse:batch. *pointer indicates the control point.

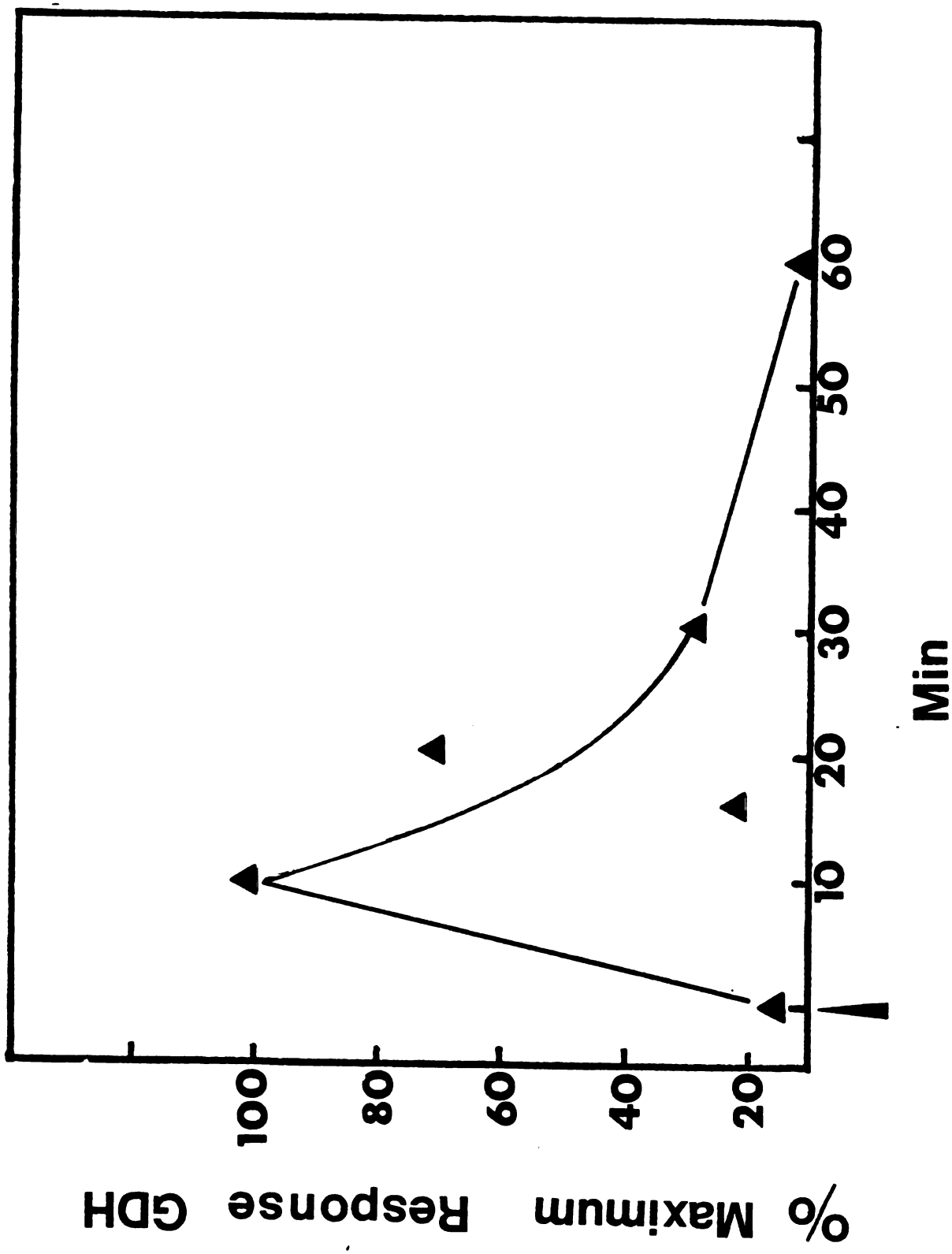
Batch cultures were grown to mid-lag phase and ammonium acetate was added (pulsed) to give a final concentration of 2 mM.

A set of duplicate cultures served as controls throughout the experiment.

Samples were withdrawn at various times after ammonium ion (ammonia) addition and assayed for glutamate dehydrogenase activity. Activities were computed as percent or fraction of maximum activity (response) measured during the course of the experiment. The obscissa represents time after ammonia addition.

***max. act:93 n moles/min/ml

Figure 3



growth rates may be viewed as a complex function of ammonia concentration. The latch phenomenon is also indicative of the higher efficiency of nitrogenase coupled ammonia supply compared to NH_4^+ dependent supply. Given the factor of latching or shifting of ammonia assimilatory systems, first-order growth rate kinetics with respect to ammonium ion concentration cannot be assumed. An attempt to derive a mathematical expression for the ammonia-dependent growth rate function yielded a cubic least-squares polynomial which best fit the observed data (Figure 2, Table 1). The ammonia-dependent growth rate function is bounded by quadratic and cubic least-squares polynomials (Appendix B). These curves are expressions of ammonium ion utilization efficiencies in the absence of nitrogen fixation and are indicative of the complexities associated with nitrogen metabolism in A. vinelandii.

The Effect of 2 mM NH_4^+ on Nitrogen Fixation and cAMP Pools in Batch Culture

Given the data of the latch study, it was important to determine the effect of 2 mM ammonia addition on nitrogenase activity in batch cultures. Nitrogenase activity declines to fifty percent of the control level upon the addition of ammonium ion (Figure 4, Table 2). After the initial decline, a quadratic least-squares polynomial best fit the behavior of nitrogenase as a function of time (Figure 4).

Figure 4.--cAMP and nitrogenase response to 2mM batch pulse.

(△) Nitrogenase activity

(□) cAMP pool level

denotes control

Dashed line approximates exponential behavior of cAMP pool response. Extrapolation from this curve yields a t for cAMP of 34 min.

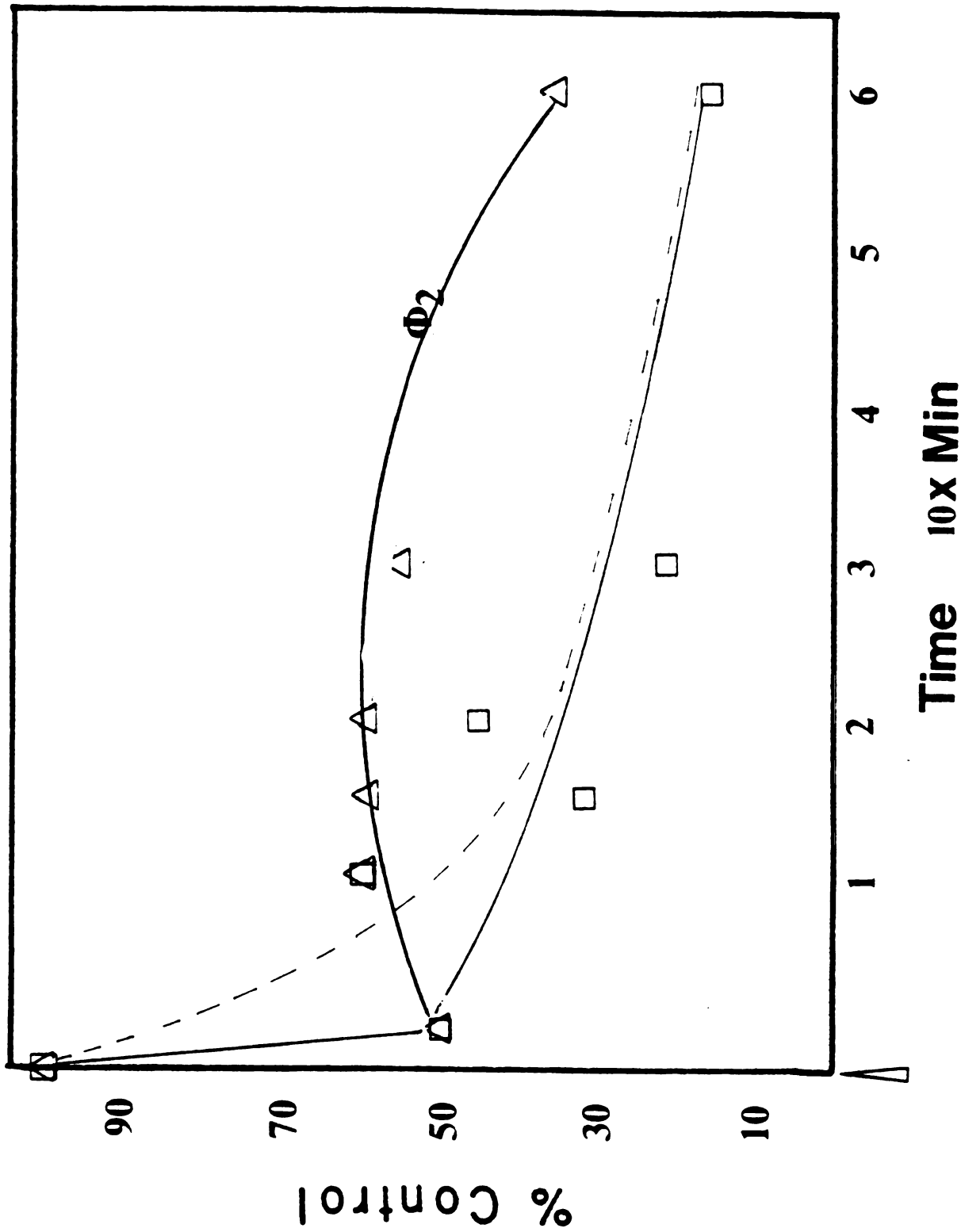
(Phi sub 2) indicates the region of the nitrogenase response curve best approximated by a quadratic least-squares polynomial.

*Control activity taken prior to ammonia addition.

Nitrogenase control = 100 n moles C_2H_2/ml culture/min.

cAMP control = 8 p moles/ml culture.

Figure 4



There has been some question regarding the mode of nitrogenase regulation in response to the addition of excessive levels of ammonia. If the mode of regulation were purely repressive, one would expect the decline in nitrogenase activity to parallel the rate of population increase. This would imply an exponential decline having a half life equal to the doubling time of the population. During the course of this study, many ammonia pulses of batch cultures were performed. In each case, the rate at which nitrogenase activity declined was far more rapid than that which could be explained by a purely repressive mechanism. It is possible, however, for a mechanism of repression coupled with dynamic protein turnover to display this sort of behavior. An inhibitory mechanism, given the rapidity of decline is equally possible.

Given evidence that ammonia is not the direct or immediate mediator of nitrogenase regulation, the possibility exists that a "second messenger" may respond to addition of ammonia. In hormonal systems of higher animals, cyclic nucleotides fit this role. The involvement of cAMP in the regulation of N_2 fixing systems has been implicated (5,6). In parallel with the nitrogenase study conducted above, changes in the cAMP pool level, upon the addition of ammonia, were also measured. The data indicate a rapid and steady decline of cAMP levels upon ammonia addition (Figure 4). The decline is best

described as exponential, such that cAMP has an estimated half life of 34 min.

Steady State Analysis

The advantage in the use of continuous culture techniques in the study of metabolic dynamics is that the organisms may be grown under definable steady state conditions. In batch culture, the steady state is not as easily defined and, in many cases, is transient in nature (Appendix A). Also, in the study of the dynamic behavior of a system it is often desirable to perturb the system from steady state and follow events concomitant with the return to that initial state. Such studies are not possible in batch culture as any perturbation from an initial steady state will result in the establishment of a new and different steady state.

In this study, two methods for the perturbation of the steady state by ammonia (ammonium ion) were employed. The first method is referred to as a "quick-pulse" where chemostatic cultures were subjected to an immediate pulse of excess ammonia. As ammonia washes out of the vessel, the conditions under which the initial steady state was established are restored (Appendix A). The wash-out rate and resident levels of ammonia are calculable and should follow first-order

kinetics (Appendix A). Deviations from first-order kinetics are due to events mediated by the organisms. The second method of perturbation of the steady state is referred to as "slow-pulse" (Appendix A). Cultures are subjected to the gradual addition of ammonia which results in the establishment of a new steady state (Appendix A). Given the property of a shift to a new steady state, slow-pulse perturbation bears some similarity to batch culture perturbation.

The dynamic behavior of nitrogenase activity under quick-pulse conditions is presented in Figure 5. The behavior of nitrogenase activity under the above conditions may best be described as a complex wave-form. This behavior is markedly different from that obtained with batch and slow-pulse perturbation (Figures 4 and 8 respectively). One interpretation of this is that the rapid and transient pulse of excessive levels of ammonia induces gross instability in the system or mechanism which regulates nitrogenase activity. The gradient effects of slow-pulse and low level batch perturbation, may permit the regulatory mechanism to exert a more gradual and stable readjustment of nitrogenase activity. This is an important finding, in that any proposed regulatory mechanism should also display this type of behavior. Under conditions of quick-pulse, the best polynomial approximation of nitrogenase behavior was found to be of fifth degree. This does not imply that the kinetics of the system are fourth-order. The polynomial, rather, serves as a

Figure 5.--Response of cAMP to quick-pulse.

See Appendices A and B for further details.

A measurement of cAMP pool level fluctuation after the addition of 10 mM NH_4^+ to the chemostat.

Data presented as percent change in cAMP pool levels with respect to the level measured in the untreated culture.

Max cAMP = 20 p mol/ml culture.

Figure 5

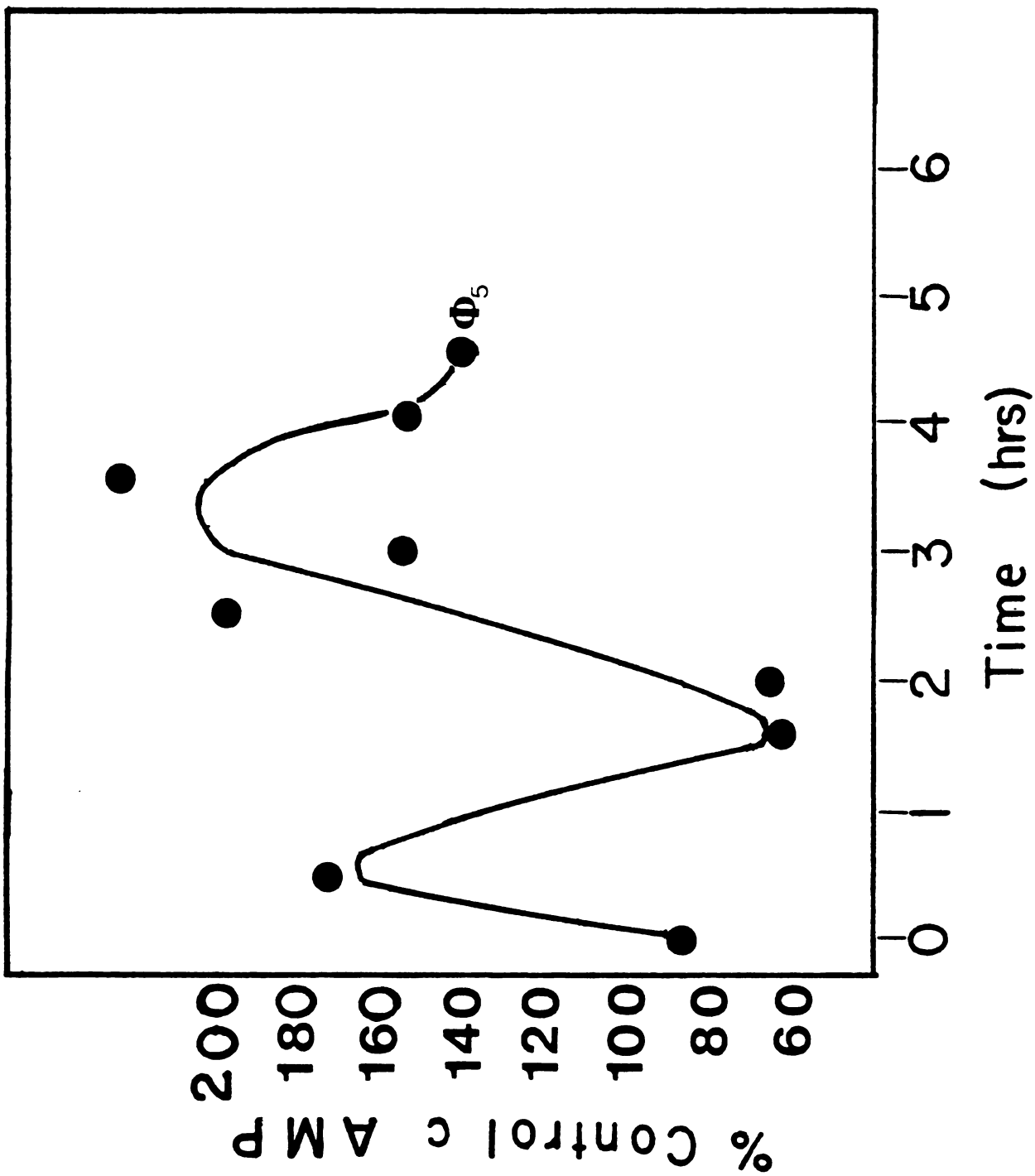

% Control c AMP
200
180
160
140
120
100
80
60
0
1
2
3
4
5
6
Time (hrs)
Φ_5

Figure 6.--Response of nitrogenase to quick-pulse.

*cross-hatched area is an artifact of polynomial regression.

**Dashed line depicts observed behavior in the interval (0, 1 h).

***Phi sub 5 indicates degree of polynomial drawn. See Appendix B.

Time zero level is that obtained immediately after ammonium ion addition.

Nitrogenase control = 150 n moles/ml/min.

Figure 6

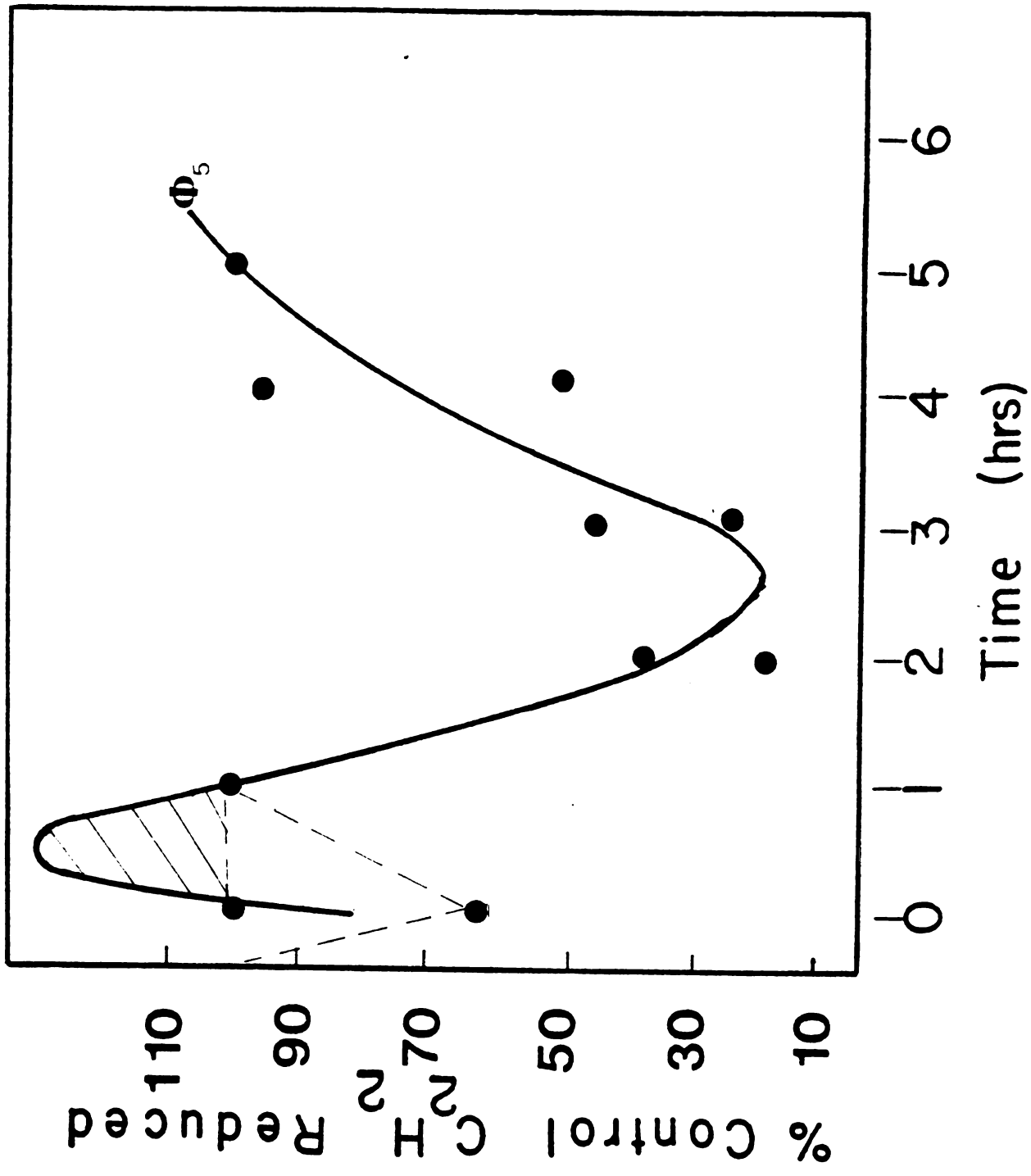


Figure 7.--Glutamine synthetase response to quick-pulse.

*Pointer indicates the control point. See Appendix B for explanation of Phi.

Control = 120μmoles/min./ml culture.

Figure 7

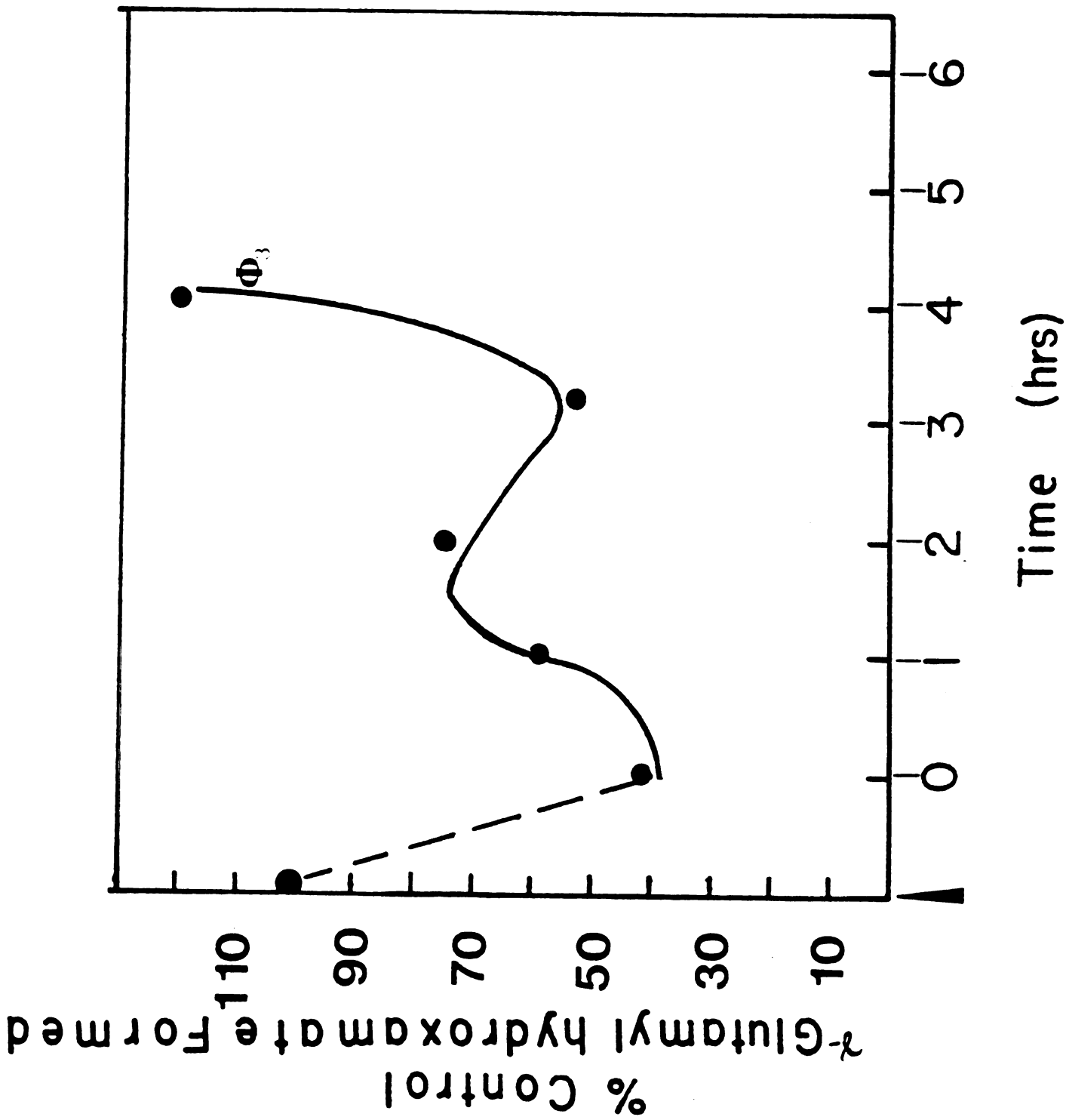

% Control
γ-Glutamyl hydroxamate Formed
110
90
70
50
30
10
0
1
2
3
4
5
6
Time (hrs)

Figure 8.--Response of nitrogenase and glutamine synthetase to slow-pulse.

**Note, the curve for glutamine synthetase is a quartic polynomial, and the portions of the curve above the 100% level are artifactual. The curve was drawn to demonstrate the hazards of extrapolation beyond the interval of approximation.

(▲) Nitrogenase activity.

(■) Glutamine synthetase activity.

Control Nitrogenase = 100 n moles C_2H /min./ml

Control Glutamine Synthetase = 75μ moles/min./ml

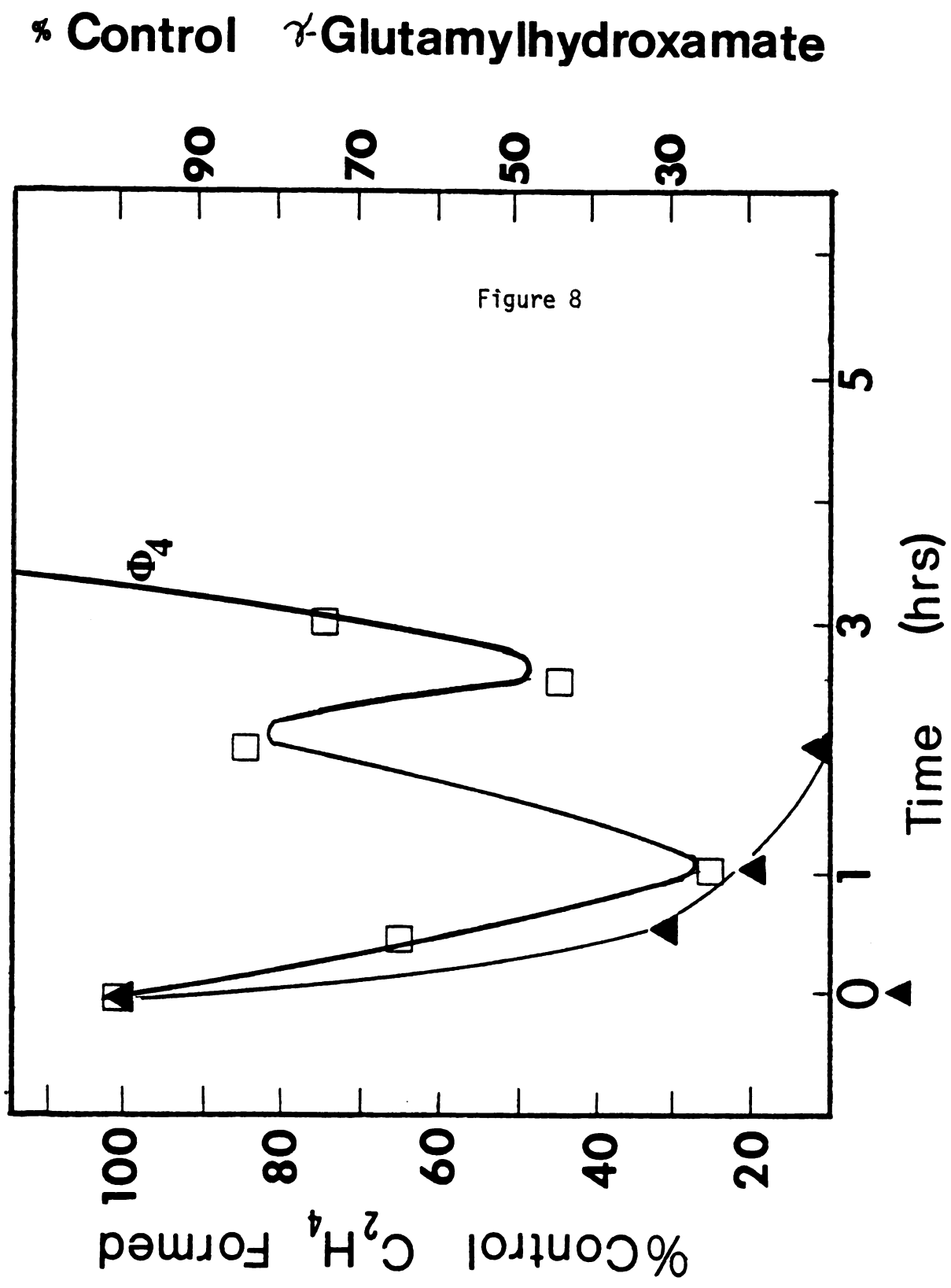

% Control
γ-Glutamylhydroxamate
90
70
50
30
Φ4
100
80
60
40
20
% Control C2H4 Formed
0
1
3
5
Time (hrs)

Figure 8

mathematical expression which best describes the observed behavior. The fact that the polynomial of best fit is of fifth degree implies extreme non-linearity in the set of parametric functions which do comprise an exact representation of the regulatory system. The polynomial, then, may be thought of as a series approximation of the summation of effects exerted by the regulatory system.

All of the parameters measured under the conditions of quick-pulse exhibited a wave-like behavior. Glutamine synthetase, cAMP, and nitrogenase were all best approximated by polynomials of degree three or higher. Superimposing the curves for nitrogenase activity, and ammonia transport reveal that the two waveforms to be in phase (Figures 5, 6, and 10). Glutamine synthetase, however, is shifted out of phase (Figure 10).

The study of resident ammonia levels during quick-pulse yielded very interesting results. Superimposition of the curves for glutamine synthetase activity and the deviation of NH_4^+ concentration indicates coincidence between the rise of glutamine synthetase activity and the beginning of ammonium uptake; occurring 1.5-2h after the pulse of ammonia (Figure 10). This may be indicative of the involvement of glutamine synthetase in ammonium transport. Also, the data derived for ammonium uptake are in close agreement with the findings made by Kleiner (4). It should also be noted that nitrogenase activity approaches its minimum as ammonium uptake approaches

Figure 9.--Ammonia uptake during quick-pulse.

See Appendix A and text for explanation.

Deviation refers to discrepancies observed upon comparison of measured ammonium ion concentrations and those predicted by "wash-outs". The time scale refers to hours after the addition of ammonium ion to the chemostat.

Figure 9

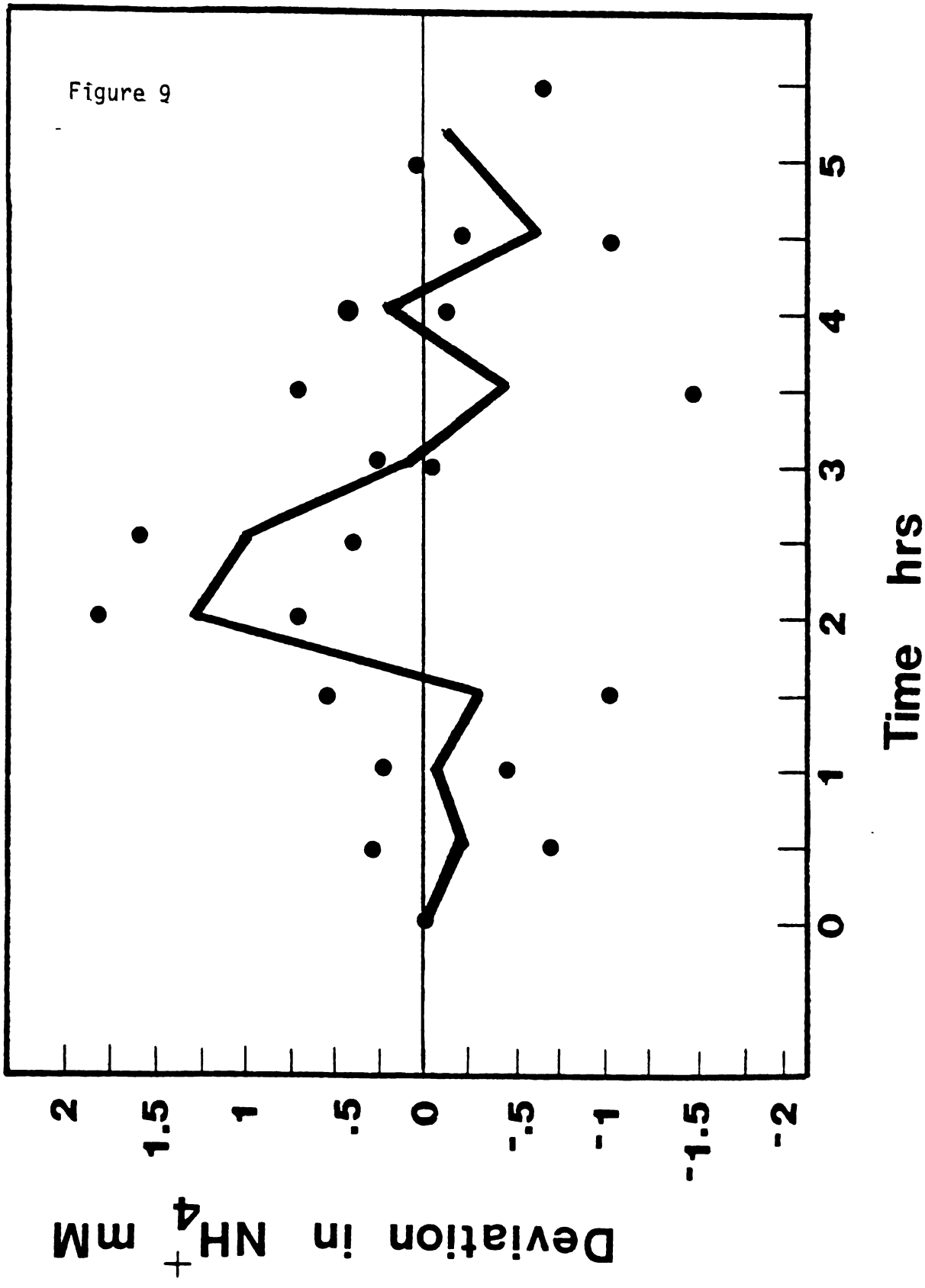


Figure 10.--Composite of nitrogenase, glutamine synthetase and ammonium transport responses to quick-pulse.

Solid line (——) glutamine synthetase activity.

Dashed line (----) ammonium transport.

(— - —) nitrogenase activity.

Curves for nitrogenase and glutamine synthetase activities represented by polynomial curves. See Appendix B for further explanation.

Left ordinate relates to nitrogenase activity and glutamine synthetase.

Right ordinate corresponds to NH_4^+ uptake.

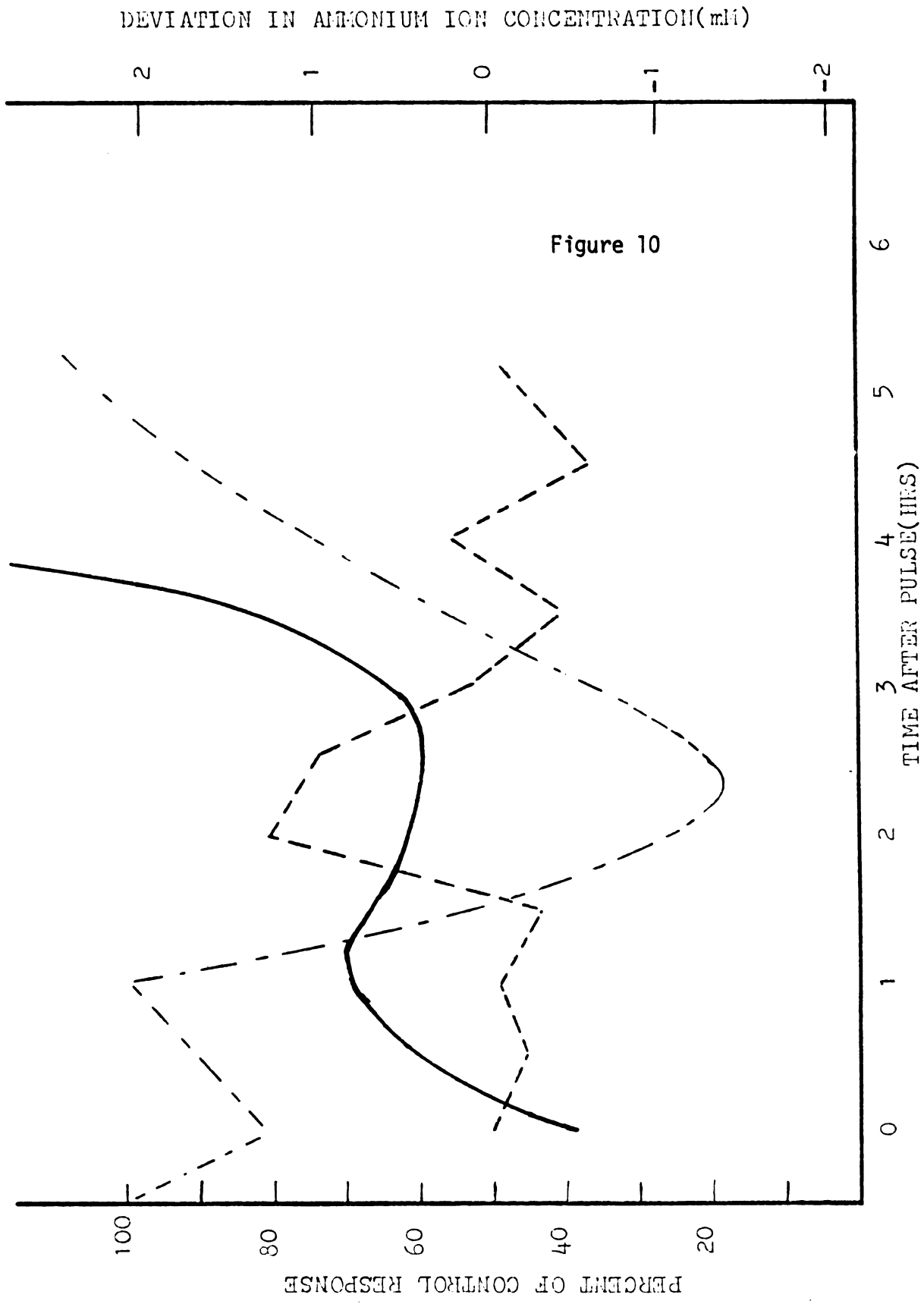

DEVIATION IN AMMONIUM ION CONCENTRATION(mM)
2
1
0
-1
-2
PERCENT OF CONTROL RESPONSE
100
80
60
40
20
TIME AFTER PULSE(HRS)
0
1
2
3
4
5
6

Figure 10

its maximum (Figures 6, 9, and 10). Glutamine synthetase exhibits the same complex behavior under both slow and quick-pulse perturbation (Figures 8 and 10). Nitrogenase activity, on the other hand, exhibits radically different behavior under the aforementioned conditions. It is, therefore, concluded that glutamine synthetase is not a direct effector of nitrogenase regulation. It is possible that elements in the glutamine synthetase regulatory cascade may be effectors in the regulation of nitrogenase. From these data, it seems that the key to the regulation of nitrogenase catalysis in A. vinelandii lies in an understanding of the ammonia transport and assimilatory systems, as there is some evidence that the two systems are one and the same.

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APPENDICES

APPENDIX A

CHEMOSTAT DYNAMICS

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The conditions for steady-state growth in batch culture are, generally, met when substrate concentrations are present in non-limiting proportions. Growth under these conditions procedes at a constant rate as defined by the logistic growth rate function:

$$\frac{dN}{dt} = \mu t$$

where μ is the intrinsic growth rate constant and N denotes population density. The exponential rate of growth, however, cannot be maintained indefinitely because at some point during growth one or more of the growth rate parameters becomes a limiting factor. The depletion of an essential nutrient, accumulation of toxic metabolic by-products, or population-mediated decline in $P(O_2)$ are all examples of events which may arise as limits on the rate of population growth. In the case of the depletion of an essential nutrient, population growth no longer procedes in the manner described by the logistic growth rate function, but, rather, in the manner described by the Monod relationship. This relationship is given by the equation:

$$\mu = \mu_{max}[S/(S+K)]$$

where μ_{max} is the rate of maximum growth at saturating levels of the essential nutrient, S. Thus, the intrinsic growth rate of the population becomes a function of the concentration of the limiting substrate. If one could precisely determine when, during the course of an experiment, any parameter becomes limiting then the principles of metabolic dynamics and the steady-state could be applied to batch culture with impunity. This is difficult, if not impossible, to accomplish by practical means. This property presents the major drawback in the application of batch culture techniques in the study of dynamic metabolic behavior. Limiting factors may alter the parameters under study long before changes in population growth rate become detectable.

One way to circumvent the problems posed by batch culture is to supply an essential substrate at a known and limiting rate with all other nutrients in non-limiting proportions. Due to the Monod relationship the growth rate of the population will be a function of the rate of supply of the limiting nutrient. Therefore, if the rate of nutrient supply is constant then the population growth rate is constant. This relationship is derived from a modification of the Monod relationship and is given by the equation:

$$D = \mu = \mu_{max} S/(K_s + S)$$

where D is the dilution factor, in units of inverse time. In practice, D is usually defined as medium flow rate per unit time/volume of

culture. As D may be defined and modified at will, so may the growth rate of the population as long as D does not exceed the maximum growth rate μ_{max}. If D exceeds μ_{max}, then wash-out of the population occurs as the rate of population growth can no longer match the dilution rate. This property is often referred to as "wash-out kinetics" and applies to any product or particle which does not accumulate at a rate greater than or equal to D, and is given by the differential:

$$\frac{dX}{dt} = -DX$$

The property of wash-out kinetics was exploited in the quick-pulse perturbation of the steady-state and the ammonia uptake study. Let C(0) be the initial ammonia concentration immediately after addition. Then the concentration of ammonia resident in the chemostat at any time, t, thereafter is given by:

$$C(t) = C(0) \exp(-Dt)$$

which is an exponential decline in the concentration. However, if ammonia is assimilated or excreted by the organisms in response to the pulse, then the observed ammonia concentration will deviate from that expected by wash-out. Let C(t) be the expected ammonia concentration and C'(t) be that which is observed. Then the deviation from expectation, D(t), at any time, t, is given by the equation:

$$D(t) = C(t)-C'(t)$$

In the calculation of the uptake data, C(t) was computed by the equation:

$$C(t) = C'(t-1)\ \exp(-Dt)$$

and

$$D(t) = C(t)-C'(t)$$

If D(t) is zero the wash-out kinetics operator applies.
If D(t) is positive--uptake.
If D(t) is negative--excretion or nonspecific absorption.

Table 1 and Figure 9 demonstrate the results of this technique in pinpointing the induction (tentative) of a late ammonia translocase.

Slow-pulse is also a midification of wash-out kinetics as influx equals efflux equals D. For slow-pulse, let C(r) be the concentration of ammonia in the medium reservoir and C(t) be defined as before. The concentration of ammonia in the chemostat vessel at any time, t, after the start of influx is given by the following equations:

$$\frac{dC}{dt} = D[C(r)-C]$$

and

$$C(t) = C(r)[1-\exp(-Dt)]$$

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APPENDIX B

POLYNOMIAL APPROXIMATION

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POLYNOMIAL APPROXIMATION

For the purposes of clarity, we shall begin this section with a brief analogy. The plotting of function graphs is a rather common experience. Given a function and a set of independent variables, the domain set (or referred to as the set of "x" values), the dependent variables or the range set can be computed and plotted. In more precise terms, one maps the domain set into the range set by means of the function. The function in this sense serves as the rule by which one may assign a value to a member in the range given the domain. For example, the function: 2x-3 indicates that for a given domain element x, its image in the range, under the mapping, is 2x-3. When functions of this sort are explicitly given, the operation of mapping is simple and straight forward. When one studies natural phenomena, graphs of dependent versus independent variables may be plotted. In many cases, the plot assumes linearity such that one may easily ascertain the function or rule which "nature" follows in the formulation of the map. However, there are many instances where the "rule" used by "nature" is not easily discernable even though there is

an intrinsic rule, function, or order applicable to observations of natural phenomena.

Numerical analysis, specifically polynomial approximation, may be employed as a technique for "breaking the code" which "nature" has imposed on the observed phenomenon. Two examples of this technique are least-squares polynomial regression (approximation) and polynomial interpolation (eg. LaGrange polynomials). The objective of both techniques is the creation of a pseudofunction which best approximates the unknown, natural function.

Interpolation is the technique whereby one attempts to derive a function which collocates or assumes the value of each data point (2). The use of interpolation polynomials ignores the variability in the observations due to statistical error (eg. errors given by a normal probability distribution). The technique also introduces artifacts at higher degrees due to the constraints of collocation (2). For these reasons, interpolation methods were not employed in this study.

Least-squares, on the other hand, fits a function to the observations in a manner designed to minimize errors due to approximation (eg. mean of squared errors from the mean). Incidentally, a least-squares polynomial can be forced to interpolate if, for n data points, one attempts to fit a least-squares polynomial of degree n-1.

Polynomials derived during the course of this study are of the form:

$$f(x) = a_0 + a_1x + a_2x^2 + a_3x^3 + \ldots + a_nx^n$$

which may be thought of as series approximations of the observed phenomena. The term, degree, refers to the largest exponent assigned to the independent variable in the polynomial. The notation P(n) or the Greek letter phi sub n denotes the degree of the approximating polynomial, and in tables denotes estimates derived from the polynomial.

The statistic, r, is termed the correlation coefficient and is a measure of the degree of change in one variable which is reflected in the change in the other(s). The correlation coefficient assumes values in the interval (-1,1), where 1 or -1 reflect perfect correlation between the two variables. The statistic, r^2, is termed the coefficient of determination or covariance, and is a measure of how well the derived function accounts for the variation within the data set or range or "y" values. In simpler terms, it is a measure of how well we have discerned "nature's rule". The coefficient of covariance assumes values in the interval (0,1); 0 implies no fit and 1 implies perfect fit. Except for growth rate studies (Figures 1 and 2) all functions derived are functions of time.

Table 1.--Ammonia Uptake * 10 mM NH_4^+ Quick-Pulse

	RUN I			RUN II		
Time (hrs.)	C'(t)	C(t)	D(t)	C'(t)	C(t)	D(t)
0	8.22	8.22	0	8.54	8.54	0
.5	7.93	7.22	-0.71	7.19	7.50	0.31
1.0	7.41	6.96	-0.45	6.03	6.31	0.28
1.5	5.96	6.51	0.55	6.35	5.29	-1.06
2.0	4.51	5.23	0.72	3.75	5.58	1.83
2.5	2.30	3.96	1.66	2.87	3.29	.42
3.0	1.70	2.02	0.32	2.56	2.52	-0.04
3.5	2.96	1.49	-1.47	1.55	2.25	0.70
4.0	2.77	2.60	-0.17	0.89	1.36	0.47
4.5	2.63	2.43	-0.20	1.80	0.78	-1.02
5.0	2.23	2.31	0.08	----	----	----
5.5	2.63	1.96	-0.67	----	----	----

Units expressed in mM NH_4

See Appendix A for description of data presentation format.

Table 2.--Ammonium ion Dependent Growth Rate: The Latch*

	GENERATION TIME (hrs.)					
NH_4 (mM)	0.5	1.0	2.0	10.0	r	r^2
Trial I	3.41	4.27	3.56	2.00		
Trial II	3.73	4.75	3.14	2.78		
P(2)	3.91	4.25	3.39	----	.845	.714
P(3)	3.57	4.50	3.35	----	.943	.889

Average Generation Time Control: 3.21h

	COEFFICIENTS			
i	0	1	2	3
a_i				
P(2)	3.07	2.19	-1.02	-----
P(3)	3.20	-0.62	3.50	-1.68

*See Text

Table 3.--Nitrogenase Activity: 2mM NH_4^+ Pulse in Batch Culture

Time (min.)	% Control C_2H_4 Formed	P(2)	i	a_i
1	50.00	53.83	0	53.31
10	60.00	51.24	1	.5256
15	60.90	58.20	2	-.0133
20	61.00	58.50	r	.8832
30	50.57	57.00	r^2	.7800
60	37.93	36.86		

Table 4.--Cyclic AMP Pool Fluctuation Response to 2 mM NH_4^+ Pulse:Batch* Culture

Time (min.)	% Control	Units**
1	50	4
10	60	4.8
15	32	2.56
20	46.62	3.73
30	22.61	1.81
60	17.38	1.39
Control	100	8.00

*See test for description.

**Units recorded as picomoles cAMP per ml culture per Klett $_{450}$ unit X 1000.

Note: The best approximation of the above response is exponential ie.

% Control = 55.31 exp(-.02/min. t) $\quad t_{1/2} = 34.65$ min.

Table 5.--Glutamine Synthetase Activity * 10 mM NH_4^+ Quick-Pulse

				Coefficients	
Time (hrs.)	Specific Activity	% Control	P(3)	i	a_i
0	48.24	40.00	37.6	0	37.60
1	71.28	59.10	68.66	1	65.20
2	91.98	76.72	61.92	2	-41.71
3	64.62	53.58	63.14	3	7.62
4	145.26	120.45	118.05	r	.9469
Control	120.60	100.00	----	r^2	.8966

P(3)--Cubic polynomial estimate

Specific activity in units of glutamylhydroxamate (umoles)/min./(ml culture).

*All data recorded to two significant figures.

Table 6.--Nitrogenase Response to 10 mM NH_4^+ Quick-Pulse*

Time (hrs.)	% Control C_2H_2 Reduced				
	RUN I	RUN II	P(5)	i	a_i
0	64	100	82	0	82
1	100	100	100	1	232.95
2	32	18	26	2	-347.92
3	45	26	35	3	161.58
4	96.8	52	75	4	-30.65
5	100	--	100	5	2.08
				r	.911
				r^2	.830

*See text for description.

Table 7.--Cyclic AMP Pool Fluctuation * 10 mM NH_4^+ Quick-Pulse

			Coefficients	
Time (hrs.)	% Control	P(5)	i	a_i
0	84.12	85.68	0	85.68
.5	171.51	167.42	1	478.21
1.5	59.64	63.70	2	-856.71
2.0	65.73	83.54	3	513.40
2.5	197.77	146.90	4	-122.86
3.0	153.86	202.12	5	10.22
3.5	222.55	204.90	r	0.8892
4.0	156.53	156.41	r^2	0.7906
Control	100.00	----		

% control computed as picomoles cAMP (sample)/picomoles cAMP (control) X 100.

All date presented to, at least, two significant figures.

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