REGULATION OF NITROGEN METABOLISM IN VIBRIO ALGINOLYTICUS

by

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Submitted in partial fulfilment of
the requirements for the degree of

M.Sc.

in the Faculty of Science

University of Cape Town
Cape Town

March 1984

ACKNOWLEDGEMENTS

My sincere appreciation and gratitude to my supervisor, Professor D.R. Woods, for his tremendous enthusiasm, energy and expert guidance. Many thanks to Associate Professor F.T. Robb for his interest and assistance at all times.

For support and encouragement, I am indebted to my good friend and colleague, Eugenia Barros. Thanks to Mrs Joan Lomberg for her willingness to help with any problem. Special thanks to my parents for their love and concern.

Much appreciation to Mrs Rose Marie Estment for typing this thesis, to C. Davis, J. Arnold and E. Barros for their help in proofreading, and to S. Fritz for the photocopying.

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ABSTRACT

Aerated cultures of Vibrio alginolyticus produced histidase at 30°C but production of histidase was repressed by either incubation at 37°C or a lack of oxygen. A similar regulation system by temperature and oxygen has been reported for collagenase and protease production by V. alginolyticus (Hare et al., 1981). V. alginolyticus had identical growth rates at 30 and 37°C.

The histidine-utilization (hut) enzymes were coordinately induced by histidine. The inducible nitrogen catabolic enzymes arginase, alanine dehydrogenase and histidase were not subject to nitrogen catabolite repression. Various amino acids and ammonium ions stimulated the production of histidase and arginase. Urocanase and formiminoglutamate hydrolase were repressed by nitrogen-containing compounds. Tryptophan, glutamine and isoleucine either repressed or had little effect on the production of histidase and urocanase. The hut enzymes and alanine dehydrogenase were sensitive to catabolite repression by glucose. The addition of $(NH_4)_2SO_4$ stimulated histidase production. Cyclic AMP did not relieve repression by glucose. Catabolite repression by glucose of collagenase and protease production in V. alginolyticus was also not relieved by cyclic AMP (Reid, 1981; Long et al., 1981).

The preferred buffer for glutamine synthetase transferase activity (GS_T) was imidazole buffer supplemented with Mg^{2+}. The optimum concentrations for the Mg^{2+}- and Mn^{2+}-dependent biosynthetic activities (GS_B) were 1.67 M and 0.1 M respectively. In crude extracts prepared from exponential phase cells of V. alginolyticus, Mg^{2+} was the preferred cation for NH_4^+-shocked and unshocked cultures. GS_B activities of crude extracts of V. alginolyticus cells grown in various nitrogen sources indicated that either Mg^{2+} or Mn^{2+} could serve as an activator. The GS_B reaction cannot be utilized for crude extracts of V. alginolyticus. The inhibition of transferase activity by 60 mM Mg^{2+} and the forward transferase reaction could not be demonstrated for the GS of V. alginolyticus.

The addition of $(NH_4)_2SO_4$ to nitrogen-limited cultures of V. alginolyticus resulted in an NH_4^+-dependent decrease in GS_T activity. This was not due to a shift in the pH optimum. The pH optimum for GS_T activity of shocked and unshocked samples of V. alginolyticus was 7.9. The GS_T activities of shocked cells were lower than those of unshocked cells at all pH values examined. Thus, no isoactivity point could be defined for the GS of V. alginolyticus.

The GS_T activity of cell-free extracts of V.alginolyticus was subject to feedback inhibition by glycine, adenosine monophosphate (AMP), tryptophan, arginine and $(NH_4)_2SO_4$ and to a lesser extent by glutamic acid, leucine, isoleucine, histidine and alanine. Feedback effects with multiple

inhibitors were antagonistic.

Growth of V. alginolyticus in various amino acids stimulated GS production. Growth in glutamine caused severe repression of GS production. The production of GS was derepressed in media containing growth rate-limiting concentrations of NH_4^+ salts. Growth of V. alginolyticus in media containing sucrose, fructose, glycerol, glucose or maltose as carbon sources resulted in the enhanced production of GS. Growth of V. alginolyticus on arabinose and lactose repressed GS production.

The synthesis of glutamate dehydrogenase (GDH) was repressed when NH_4^+ salts were present in excess. Glutamate synthase (GOGAT) production was repressed under conditions of nitrogen excess and derepressed when nitrogen was limiting. Growth of V. alginolyticus in high concentrations of glutamate resulted in low levels of GDH and GOGAT. Glucose repressed the production of GOGAT but had no effect on the levels of GDH under conditions of nitrogen limitation. Ammonia assimilation in V. alginolyticus occurred via the GS-GOGAT pathway when nitrogen was limiting, and by GDH when nitrogen was present in excess.

CHAPTER I

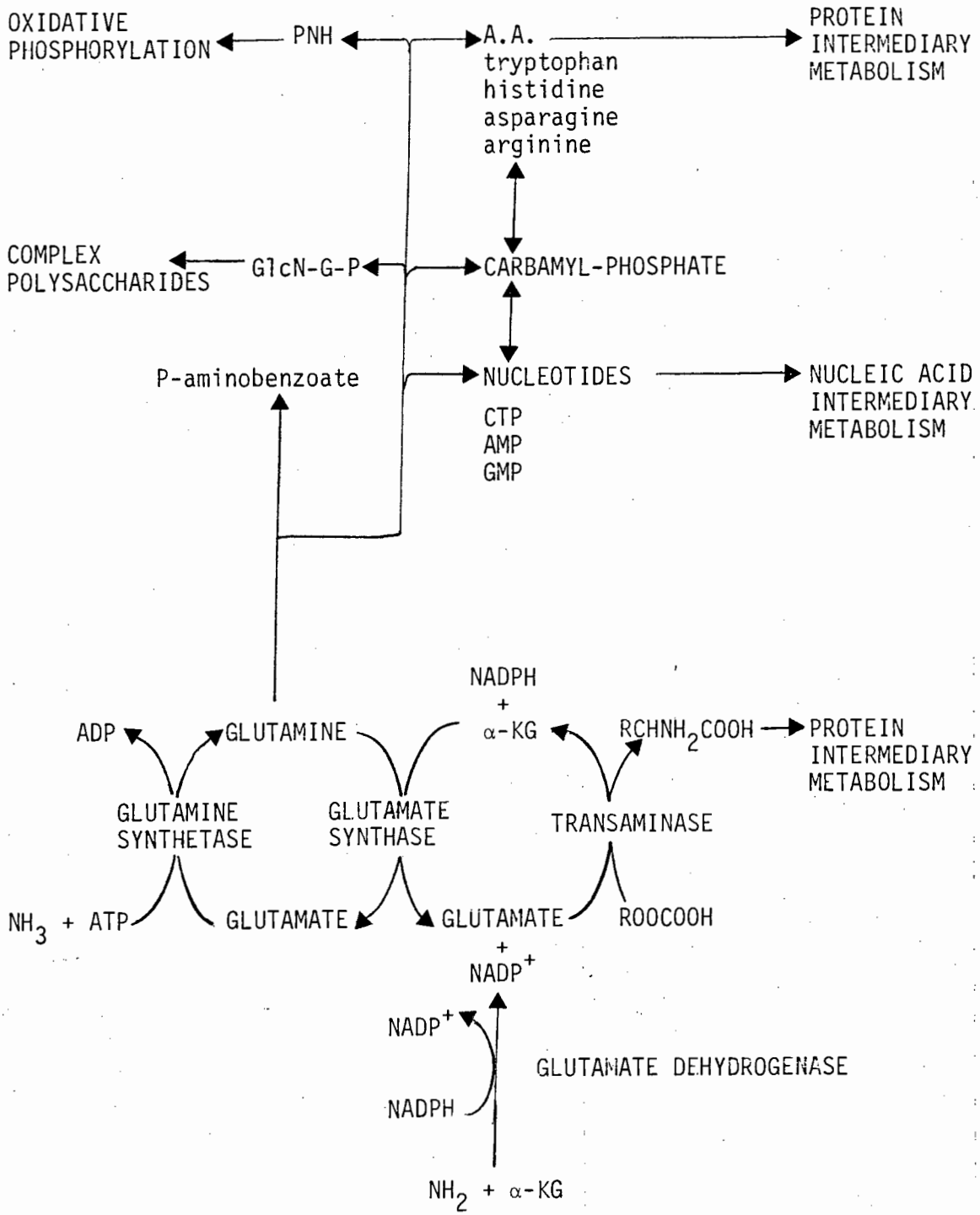
INTRODUCTION

1.1. GENERAL

Nitrogen is one of the major elements found in many of the simple compounds and nearly all of the complex macromolecules of living cells; proteins and nucleic acids are rich in nitrogen. Thus, it is not surprising that a substantial cellular investment is made in the metabolic machinery comprising the nitrogen assimilatory pathway to ensure a constant nitrogen supply for growth. Ammonia, glutamate and glutamine are the favoured nitrogen sources (Fig. 1.1). Microorganisms, however, possess the ability to utilize diverse secondary sources including nitrate, nitrite, purines, proteins and numerous amino acids. Use of these secondary nitrogen sources requires the synthesis of catabolic enzymes or the activation of previously existing enzymes.

The metabolic pathways of nitrogen metabolism can be divided into two classes: the assimilatory pathway necessary for the utilization of nitrogen from compounds in the medium and the biosynthetic pathways leading to the production of nitrogen-containing compounds in the cell. The exact array of usable nitrogen compounds is a characteristic of each organism and the specific steps in these pathways vary with the organism. In virtually all cells glutamate and glutamine serve as the nitrogen donors for biosynthetic reactions.

FIG. 1.1. PATHWAYS OF AMMONIA ASSIMILATION IN THE ENTERIC BACTERIA FOR THE PRODUCTION OF GLUTAMATE AND GLUTAMINE, AND SOME OF THE ROLES OF THESE COMPOUNDS IN INTERMEDIARY METABOLISM (TYLER, 1978).



A knowledge of the formation of glutamate and glutamine from various nitrogen sources is crucial to understanding cell growth.

Two alternate routes exist in bacteria for the conversion of ammonia to glutamic acid. They are mutually exclusive. At low ammonia concentrations, glutamate synthase (GOGAT) acts with glutamine synthetase (GS) in catalyzing the adenosine triphosphate (ATP)-dependent conversion of ammonia and α-ketoglutarate (α-KG) to glutamate.

$$\text{Glutamate} + \text{ATP} + NH_3 \xrightarrow{\text{GS}} \text{glutamine} + \text{ADP} + P_i + H_2O$$

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

$$\alpha\text{-KG} + NH_3 + \text{NADPH} + H^+ \xrightarrow{\text{GS}} \text{glutamate} + \text{ADP} + P_i + NADP^+ + H_2O$$

In the absence of ATP and when the above pathway is inactive, the alternate pathway, catalyzed by glutamate dehydrogenase (GDH) is used.

$$\alpha\text{-KG} + \text{NADPH} + H^+ + NH_3 \xrightarrow{\text{GDH}} \text{glutamate} + NADP^+ + H_2O$$

The cell does not require GS for the production of glutamate in a medium containing a sufficiently high ammonia concentration for GDH to operate efficiently. Conversely, the cell does not require GDH when the exogenous ammonia concentration is too low for its activity. Bacterial cells have thus evolved appropriate control mechanisms for the synthesis of these enzymes.

1.2. FORMATION OF GLUTAMATE AND GLUTAMINE FROM AMMONIA

The major part of this section is taken from Tyler (1978).

1.2.1. THE ROLE OF GLUTAMATE DEHYDROGENASE

BIOCHEMISTRY OF GDH: GDHs catalyse the reductive amination of α-KG by ammonia in a reversible reaction, that requires either nicotinamide adenine dinucleotide phosphate (NADPH) or NADH. These enzymes occur in a wide variety of organisms. In microorganisms, NAD-dependent GDHs appear to serve a catabolic function, while the enzymes that utilize NADPH serve chiefly for the biosynthesis of glutamate. The GDHs of Escherichia coli and Salmonella typhimurium have MWs of 300 000 and 280 000 respectively. The E. coli enzyme consists of six identical subunits. The main physiological role of this enzyme has been thought to be biosynthetic. However, due to the high Km for ammonia of these enzymes, it appears unlikely that they function efficiently in ammonia assimilation, except when the environmental ammonia concentration is high.

PHYSIOLOGY OF GDH PRODUCTION: GDH is unlikely to play a catabolic role in E. coli since growth in the presence of glutamate results in repression of this enzyme. Such regulation would be predicted if GDH serves only a biosynthetic function and would not be expected if the enzyme played any role in the degradation of glutamate. It is interesting that the GDH of Salmonella is not repressed by glutamate (Brenchley et al., 1975).

GENETICS OF GDH FORMATION: GDH mutants of E. coli, Klebsiella aerogenes or S. typhimurium can grow in the absence of glutamate, thus confirming the existence of an alternative pathway for the biosynthesis of glutamate. These mutants grow as readily as wild-type (WT) strains on other nitrogen sources. The role of GDH in ammonia assimilation can therefore be completely replaced by the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway.

1.2.2. THE ROLE OF GLUTAMATE SYNTHASE

BIOCHEMISTRY OF GOGAT: Glutamate synthase is abbreviated GOGAT from its previous trivial name: glutamine amide α-ketoglutarate aminotransferase (oxidoreductase, NADP). GOGAT is one of a number of enzymes that catalyse the transfer of the amide group of glutamine in various biosynthetic reactions. The reaction catalyzed by GOGAT, where glutamine reacts with α-KG to form two molecules of glutamate, was discovered by Tempest and co-workers (1970). They found that nitrogen-limited cultures of K. aerogenes were able to grow in a glucose-ammonia-mineral medium in cells lacking GDH. The presence of GOGAT is well-established in the prokaryote kingdom. Aspartase and alanine dehydrogenase were thought to be the possible catalysts for reactions leading to ammonia assimilation in bacteria lacking GDH.

GOGAT, purified from E. coli and K. aerogenes is an iron-sulfide flavoprotein, consisting of a dimer of two unequal subunits. The smaller subunits from both bacterial species

have the same MW of 53 000 but the larger polypeptide from K. aerogenes is heavier (MW 175 000) than that from E. coli (MW 135 000). The subunit composition of intracellular GOGAT is not clear. Although GOGAT and GDH are functionally similar, (effecting synthesis of glutamate from α-KG by an oxido-reduction reaction), they are very different enzymes. Apart from having different substrate requirements, (glutamine versus ammonia, as the amino-donor molecule), the GOGAT-mediated reaction is virtually irreversible and completely inhibited by a glutamine analogue, 6-diazo-5-oxo-L-norleucine (DON) while it had no effect whatsoever on the GDH reaction (Tempest et al., 1970).

REGULATION OF GOGAT SYNTHESIS AND ACTIVITY: With ammonia-limited and glucose-limited cultures of K. aerogenes, a reciprocal relationship seemingly existed between the cellular contents of GOGAT and GDH. Conditions that favoured the synthesis of GOGAT caused suppression of GDH synthesis, and vice versa. In fact, with all the bacterial species so far examined, ammonia limitation invariably caused repression of GDH synthesis and promoted synthesis of GOGAT. Some organisms, Erwinia carotovora, Bacillus subtilis W23 and Bacillus megaterium, lacked GDH, but could still grow readily in a simple salts medium in which ammonia provided the sole source of utilizable nitrogen; in these cases GOGAT was synthesized constitutively.

Although it is not possible to make definitive statements about GOGAT regulation from any of the work done so far, it

is clear that synthesis of GOGAT is regulated. However, many questions remain to be answered. Is there a specific repressor or activator regulating transcription of the glt B gene, a gene involved in GOGAT biosynthesis in E. coli? How is synthesis of GOGAT affected? What is the role of glutamate in the regulation of GOGAT?

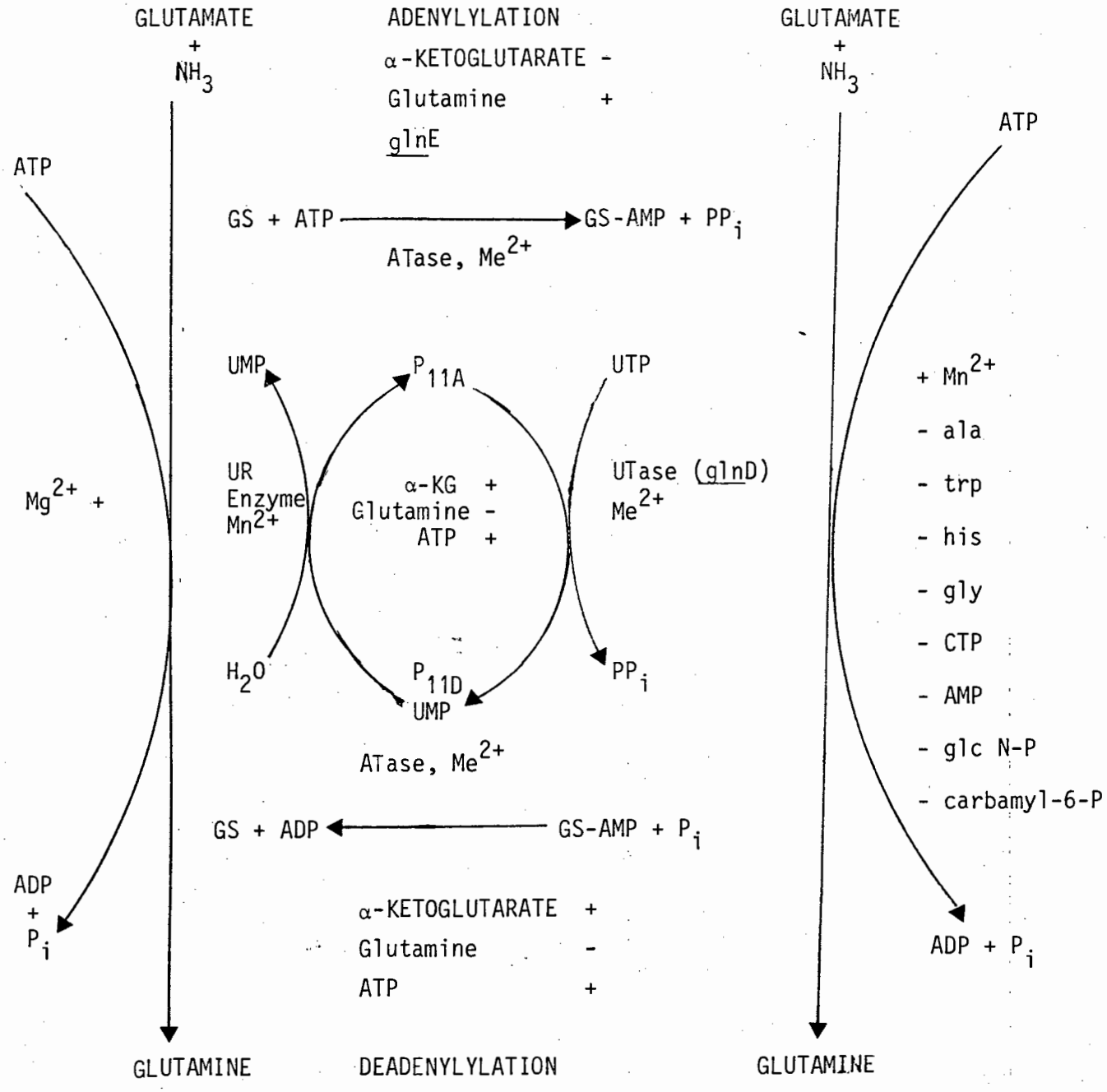
GENETICS OF GOGAT FORMATION: GOGAT mutants of K. aerogenes, E. coli and S. typhimurium do not require glutamate for growth on mineral salts medium containing glucose and excess ammonia. However, in contrast to gdh mutants, strains lacking GOGAT activity have a distinct phenotype. These mutants are unable to grow on glucose minimal medium containing a low concentration of ammonia or a variety of other nitrogenous compounds as the sole source of nitrogen. Therefore, they were originally described as ASM$^-$ strains (ammonia assimilation negative strains).

1.2.3. THE ROLE OF GLUTAMINE SYNTHETASE

GS catalyses the ATP-dependent production of glutamine from ammonia and glutamate, a highly significant reaction in nitrogen metabolism, for glutamine serves as the nitrogen donor in the biosynthesesof diverse nitrogenous metabolites, including histidine, tryptophan, carbamyl-phosphate, glucosamine-6-phosphate, adenosine monophosphate (AMP) and cytosine triphosphate (CTP) (Fig. 1.2). Thus, GS occupies a strategic branch-point in cellular metabolism. This consideration suggested that it was a likely target for cellular control and has provoked extensive studies to ascertain its regulatory mechanism.

FIG. 1.2: ADENYLYLATION AND DEADENYLYLATION OF GLUTAMINE SYNTHETASE

Adapted from Ginsburg and Stadtman (1973).



Where: gln = genes regulating production
ATase = Adenylyltransferase
UTase = Uridylyltransferase
UR = Uridylyl-removing enzyme
Me^{2+} = Metal cation

FIG. 1.2: Adenylylation and deadenylylation of GS results from the interaction of P_{11A} or P_{11D} with ATase and GS as described in the text. These reactions are stimulated, +, or inhibited, - , by the levels of various metabolites and by Mg^{2+} or Mn^{2+}. The interconversion of P_{11A} and P_{11D} by UTase and UR enzyme responds to the levels of these same metabolites. The UTase is also activated by either Mg^{2+} or Mn^{2+} while the UR enzyme is activated only by Mn^{2+}.

BIOCHEMISTRY OF GS: The native enzyme purified from E. coli W has a molecular weight of 600 000 daltons and contains twelve identical subunits, which are molecularly arranged in a double hexagon, layered with a 4.5 nm spacing between them. In addition, the divalent cation Mg^{2+} or Mn^{2+} is required for stability.

REGULATION OF GS ACTIVITY: GS activity in E. coli can be regulated by at least five different mechanisms:

(i) repression and derepression of enzyme synthesis in response to variations in the nitrogen supply of ammonium salts to the cell,

(ii) cumulative feedback inhibition by multiple end-products of glutamine metabolism,

(iii) interconversion of active (taut) and inactive (relaxed) forms of the enzyme in response to fluctuations in the concentrations of divalent cations,

(iv) modulation of catalytic potential and divalent cation specificity by enzyme-catalysed adenylylation and deadenylylation of the enzyme, and

(v) cascade control of the adenylylation and deadenylylation reactions.

(i) REPRESSION OF GS FORMATION

The different roles of the glutamine synthetase of enteric bacteria are reflected in the way enzyme level and activity

respond to the nitrogen source of the growth medium. When nitrogen is readily available, the GDH pathway is utilized and GS and GOGAT are repressed. The absolute level of GS in these cells is inversely related to the availability of nitrogen in glucose minimal medium (Bender et al., 1977; Bloom et al., 1977; Senior, 1975).

During growth under continuous steady-state cultural conditions, the level of GS is a complex function of both carbon and nitrogen availability. Significantly, under conditions of carbon limitation, GS formation is not fully repressed, even in the presence of moderately high ammonium ion concentrations. Even greater levels of enzyme can be obtained if growth on glucose in continuous culture is limited by availability of glutamate as the sole nitrogen source. These studies indicate that ammonium itself is probably not the only effector regulating the synthesis of GS. Perhaps enzyme synthesis is regulated by a system of repression in which ammonium or a metabolic derivative (namely, glutamine), together with other metabolites are synergistic corepressors. Brenchley et al. (1975) found that cells grown in glucose minimal medium supplemented with certain amino acids; or in minimal medium with other carbon sources (Bender and Magasanik, 1977), contain extremely low levels of highly adenylylated GS. Bender and Magasanik (1977) have suggested that the extremely low rate of GS synthesis in cells grown in such media reflects the ratio of glutamine to α-keto-glutarate. This follows from the proposal that the absolute level of GS in WT cells reflects the adenylylation state of

the enzyme (Foor *et al.*, 1975; Gaillardin and Magasanik, 1978). The variation in the level of GS in response to changes in the environment of the cell is the result of regulation of transcription of the structural gene (*gln* A) for GS. The enzyme level reflects the intracellular concentration of mRNA complementary to *gln* A - specific DNA (Weglenski and Tyler, unpublished observation). GS was thought to play a role in the regulation of transcription of *gln* A. However, recent evidence has shown that nitrogen control is far more complex than was first realised.

(ii) CUMULATIVE FEEDBACK INHIBITION

Attention focused on GS as a central target for cellular regulation when studies of Woolfolk and Stadtman (1964, 1967) showed that GS from *E. coli* is susceptible to feedback inhibition by products of glutamine metabolism. It seems highly significant that six of the eight inhibitors, namely L-tryptophan, L-histidine, AMP, CTP, glucosamine 6-phosphate and carbamyl-phosphate are compounds whose biosyntheses utilize the amide group of glutamine. In the case of alanine and glycine, the amide nitrogen is indirectly derived from glutamine. Inhibition of GS by these metabolites is therefore the basis of a feedback regulation system in which each end-product of a highly branched pathway inhibits the first common enzyme in the pathway. These inhibitors are generally independent in their action, thus the activity of the enzyme is progressively decreased by increasing the number of inhibitors. Woolfolk and Stadtman (1964) refer

to this type of multiple inhibition as cumulative feedback inhibition. The effects of multiple inhibitors are cumulative, provided that each inhibitor is present at a physiological concentration that produces only partial inhibition by itself (Shapiro and Stadtman, 1970). Cumulative effects are readily explainable by the existence of separate non-interacting binding sites on the enzyme for almost each different feedback inhibitor. When many different end-products are derived from a common precursor, cumulative feedback inhibition of the enzyme catalysing the synthesis of the precursor compound is an effective means of regulation, since an excess of any one of the end-products will inhibit only a part of the enzyme activity; yet, when all end-products are present in excessive concentrations, synthesis of the common precursor will be almost completely inhibited. The biosynthetic activity of adenylylated enzyme was more sensitive than the unadenylylated form to feedback inhibition by tryptophan, histidine and CTP; whereas glycine and alanine were more effective inhibitors of the unadenylylated enzyme. Studies with mixtures of inhibitors are also complicated by the fact that the divalent cation and substrate concentrations determine the response of the different enzyme forms to individual inhibitors. The mechanism of these effects, as well as the nature of interaction between inhibitors, remains obscure.

(iii) TAUT, RELAXED AND TIGHTENED FORMS OF GS

When *E. coli* GS is isolated in the presence of Mn^{2+}, the enzyme is in a highly stable, catalytically active, so-called

"taut" configuration. Taut GS is resistant to disaggregation by various protein denaturants, has buried sulfhydryl and aromatic residues, and behaves as a compact, almost spherical molecule in solution. Upon removal of the divalent cations the enzyme is converted to an unstable, catalytically inactive relaxed configuration, having the same MW but a less compact structure. The protein becomes susceptible to disaggregation by a variety of agents. It is reactivated by preincubation with either Mn^{2+}, Mg^{2+} or Ca^{2+}. Reactivation is accompanied by burial of the exposed sulfhydryl groups and aromatic amino acid residues and leads to a so-called "tightened" form of the enzyme, which appears to be identical with the taut form except in that it has a tendency to undergo intermolecular aggregation and crystallise out of dilute salt solutions. It seems likely that the tightened form represents a metastable state of the enzyme that arises from the sudden addition of divalent ions to the relaxed enzyme.

The fact that divalent cations play such an important role in the structure and activity of GS, as well as the fact that the divalent cation required for the catalytic process varies with the extent of adenylylation of the enzyme, suggests that interconversion of relaxed (inactive) and taut (active) forms of GS may play a specific regulatory role. Whether such a phenomenon has physiological significance in cellular regulation remains to be determined, especially in the absence as yet of information on the intracellular concentrations of divalent cations under various nutritional and metabolic conditions. However, under conditions of marginal divalent

cation availability as might occur with accumulation of metabolite chelators such as nucleotide polyphosphates, citrate and organic phosphate esters, the concentrations of divalent cations could be reduced to levels that permit relaxation (inactivation) of the enzyme.

(iv) DIVALENT CATION CONTROL

DIVALENT CATION SPECIFICITY: Various divalent cations differ markedly in their ability to either support or inhibit the catalytic activities of adenylylated and unadenylylated enzymes. This and the complex effects of mixtures of two or more divalent cations on the pH-activity profiles and on the saturation functions and the apparent K_m values for various substrates indicate that each divalent cation stabilizes a uniquely different conformation of the enzyme. Mg^{2+} and Mn^{2+} have markedly different effects on catalytic parameters of the enzyme. Biosynthetic activity of fully unadenylylated enzyme designated E_0 requires the presence of Mg^{2+}, whereas the fully adenylylated enzyme (E_{12}) specifically requires Mn^{2+} ions.

EFFECT OF THE ATP : DIVALENT CATION RATIO: Activity of adenylylated GS is a complex function of the ATP and Mn^{2+} concentrations. When the ATP concentration is increased in the presence of a fixed level of Mn^{2+} ions or when the concentration of Mn^{2+} is increased at a fixed ATP level, the biosynthetic activity of GS increases to a maximal value and then declines. In either case, maximal activity at pH 7.0

is obtained when the ratio of ATP to Mn^{2+} is approximately 1.0; the ATP : Mn^{2+} ratio required for maximal activity is, however, a function of the pH. The ATP : Me^{2+} ratio is less critical when Mg^{2+} is used for assaying unadenylylated GS; whereas activity is inhibited by a high ATP : Mg^{2+} ratio, there is no significant inhibition by a large excess of Mg^{2+} ions. Fluctuations in the ratio of ATP to Me^{2+} as affected by the balance between oxidative phosphorylation and biosynthetic processes might therefore be an important mechanism of cellular control.

(v) THE ADENYLYLATION AND DEADENYLYLATION ENZYME SYSTEM

A METABOLITE REGULATED CASCADE ENZYME SYSTEM: GS exists in the cells in two different forms which are interconvertible via a dynamic equilibrium. Each of the subunits can be individually adenylylated or deadenylylated. The mechanism of activation and inactivation of the GS molecule has been proposed by Ginsburg and Stadtman (1973); illustrated in Fig. 1.2. The site of adenylylation in GS is a specific tyrosyl residue which is found in each subunit polypeptide chain of native dodecamer. Deadenylylation occurs by a phosphorolytic cleavage of the stable 5'-adenylyl-O-tyrosyl derivative. Heterologous interactions occur between adenylylated and deadenylylated subunits in hybrid GS molecules. Both homologous and heterologous subunit interactions influence activity, with each subunit of GS being potentially active in catalysis.

The attachment and removal of the covalently bound AMP residues from GS is accomplished by the action of an adenylyltransferase (ATase), a monomer, having a MW of 130 000. The enzyme is stimulated by glutamine and inhibited by α-KG. When the relative concentrations of α-KG is high, the deadenylylation activity of ATase is increased; when the relative level of glutamine is high, the adenylylation reaction is favoured. In addition, ATP stimulates the ATase to deadenylylate.

Since both adenylylation and deadenylylation reactions are catalyzed by the same enzyme, coupling between them must be prevented by an appropriate control of each function. A futile cycle is prevented from occurring by an elaborate regulatory system involving metabolite control of the adenylyltransferase (Table 1.1) and an interaction of ATase with a small regulatory protein, P_{11}, which exists in two interconvertible forms (Fig. 1.2).

The unmodified form, P_{11A}, stimulates only the adenylylated reaction and the modified form, P_{11D} (P_{11}UMP), stimulates only the deadenylylated reaction. In the conversion of P_{11A} to P_{11D}, a uridylyl group from UTP is covalently bound to the P_{11} protein. This conversion is catalysed by a specific uridylyltransferase (UTase) and requires also the presence of ATP, α-KG and either Mg^{2+} or Mn^{2+}; moreover it is inhibited by glutamine and inorganic phosphate (P_i). Sensitivity of the uridylylation reaction to glutamine inhibition is a function of the levels of both ATP and UTP. UTase possesses

TABLE 1.1. SOME CATALYTIC PROPERTIES OF ADENYLYLTRANSFERASE

(Ginsburg and Stadtman, 1973)

Activity	Requirements	Positive Effectors	Negative Effectors
Adenylylation	Mg^{2+} or Mn^{2+}	Gln	α-KG
(Optimum pH=8)	ATP, GS	P_{11A}	UTP
Deadenylylation	Mg^{2+} or Mn^{2+}	P_{11D} with	-
(Optimum pH=7.2)	P_i, GS(AMP)	α-KG, ATP	
PP_i-ATP Exchange	PP_i, ATP		
1% of A - activity	Mg^{2+}, GS, GLN	-	-

another activity that catalyses removal of uridylyl groups from P_{11D}, converting it back to P_{11A}. Collectively, the ATase, UTase, UR-enzyme and P_{11} protein comprise a highly sophisticated system that mediates the allosteric regulation of GS by a number of metabolites, including ATP, α-KG, glutamine and P_i.

CASCADE CONTROL: The uridylylation-deuridylylation and the adenylylation-deadenylylation reactions are the basis of two oppositely directed cascade systems, leading on the one hand to activation of GS and to its inactivation on the other. The inactivation cascade (shown in Fig. 1.3A) begins with the UR-enzyme-catalysed deuridylylation of P_{11}UMP to form P_{11A}, which in turn stimulates ATase to catalyse the adenylylation of GS, thereby converting it from a Mg^{2+}-dependent, more active form with a pH optimum at 8.0 to a Mn^{2+}-dependent, less active form with a pH optimum at 6.9. Running counter to this is the activation cascade system depicted in Fig. 1.3B. This is initiated by metabolite activation of UTase which catalyses the uridylylation of P_{11}. The P_{11}UMP then stimulates the ATase-catalyzed deadenylylation of GS, converting it back to the active form.

It is generally acknowledged that cascade systems are in principle very effective mechanisms of cellular control because they consist of a series of reactions in which one catalytic agent acts upon another, and this can lead to an amplification of primary stimuli on the activity of the last enzyme in the cascade. In addition, however, cascade

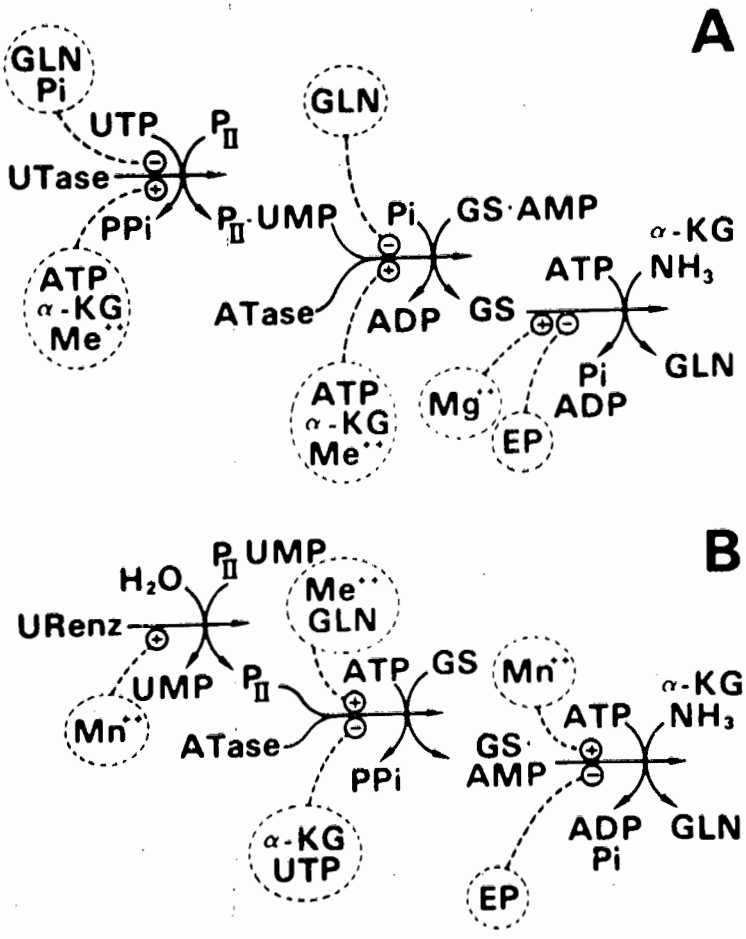


FIG. 1.3: CASCADE CONTROL OF GS ACTIVITY:

(A) GS adenylylation (inactivation) and (B) GS deadenylylation (activation). Abbreviations are as follows: EP for end-products of glutamine metabolism; plus signs, stimulation; and minus signs, inhibition.

systems increase the allosteric control potential of the regulatory system since each enzyme in the cascade is an independent target for allosteric interaction that will affect ultimately the activity of the last enzyme in the series. This increase in allosteric input potential could be important in the regulation of enzymes such as GS that occupy central positions in metabolism. In addition to their capacity to amplify regulatory stimuli and to increase allosteric input potential, cascade systems can be more finely regulated when the same effector acts at more than one step in the cascade; for example, if a given concentration of metabolite causes 50% inhibition of each of two steps in the cascade, the overall inhibition will be 75%. If a given modifier stimulates two different steps in the cascade, the apparent reaction order is increased, and stimulation of the last enzyme in the cascade will be a sigmoidal function of the modifier concentration, providing threshold responses which are of significance in cellular regulation. It is evident from Fig. 1.3 that both of the above refinements may be important in control of GS activity since two steps in the cascade, the uridylylation of P_{11} and the deadenylylation of GS, are inhibited by glutamine and are stimulated by both ATP and α-KG.

REGULATION OF NITROGEN METABOLISM

THE COMPLEX glnALG OPERON

Unless otherwise stated, most of this information is taken from the works of Tyler (1978) and Magasanik (1982).

In *E. coli* and related enteric organisms, the expression of *gln*A, the structural gene for GS, and of genes coding for enzymes capable of providing the cell with ammonia, is regulated according to the quality and abundance of the nitrogen source. This regulation is complex and involves the interaction of the products of at least five genes: *gln* B, *gln* D, *gln* F (*ntr* A), *gln* G (*ntr* C) and *gln* L (*ntr* B). Genes *gln* A, *gln* L and *gln* G are part of a complex *gln* ALG operon (Fig. 1.4) and occupy a contiguous region of approximately 4 500 base pairs. All three genes on the *E. coli* chromosome are transcribed in a counterclockwise direction from *gln* A towards *gln* G (Ueno-Nishio *et al.*, 1983). The nitrogen regulatory locus ("*gln* R") consists of two cistrons *ntr* B and *ntr* C in *S. typhimurium*, and *gln* L and *gln* G in *E. coli* (Fig. 1.4). These two genes are contiguous with the GS structural gene, *gln* A, and this close linkage confounded the earlier analysis of mutants (Merrick, 1982).

THE gln GENES AND THEIR PRODUCTS

The phenotypes and gene products of the *gln* system are summarised in Tables 1.2 and 1.3.

glnA: This is the structural gene for GS and mutations in this gene result in the Gln^- phenotype.

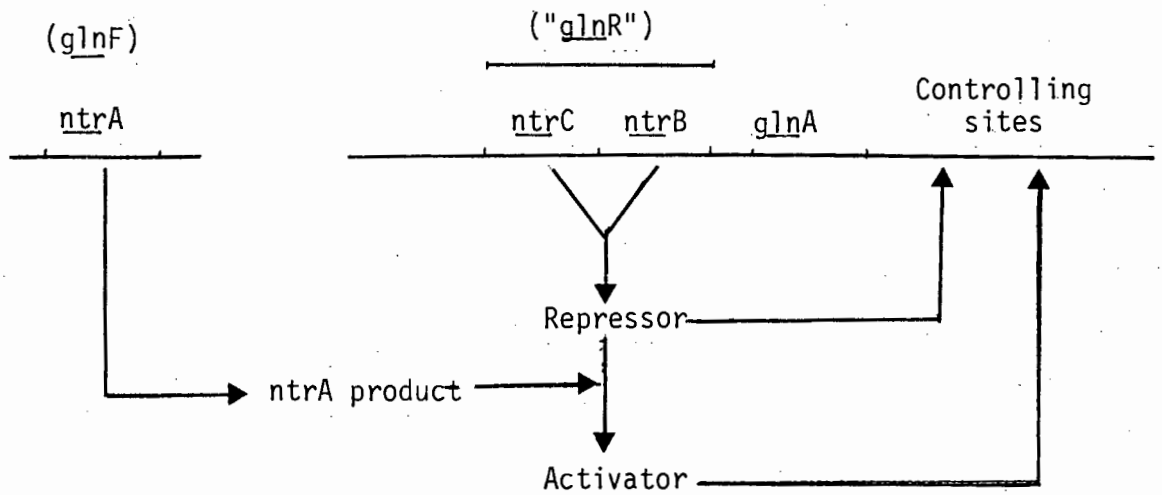


FIG. 1.4: Working model for nitrogen control in S. typhimurium and E. coli. The ntr B (gln L) and ntr C (gln G) products can repress or activate transcription of gln A and other genes under nitrogen control. The ntr A (gln F) gene product leads to the formation of an activator. The model accommodates the possibility that the ntr B (gln L) and ntr C (gln G) products may function as a protein complex (McFarland et al., 1981; Merrick, 1982).

gln F: This mutation is unlinked to gln A but also results in the Gln^- phenotype. The gln F product appears to be required for the activation of expression of the gln A gene, and for the activation of the expression of genes for ntr enzymes under conditions of ammonia starvation.

gln G: Mutations in gln G result in either low, unregulated production of GS or in GS levels lower than those found in mutants totally deficient in the gln G product. These results suggest that the gln G product can behave as an activator and repressor respectively of gln A expression.

gln D: These mutants confer the inability to produce UTase, the enzyme required for the interconversion of P_{11} and $P_{11}{}^{UMP}$ Mutants produce reduced levels of GS and the enzyme tends to be in a highly adenylylated form.

gln B: This mutant possesses a phenotype similar to that of the gln D mutant (Gln^-, Ntr^- and overadenylylated GS). The gln B gene is thought to be the structural gene for P_{11}. The gln B mutant of K. aerogenes produced an altered P_{11} which could not be converted by UTase to the form capable of stimulating the deadenylylation of GS. The role of the products of gln D (UTase) and of gln B (P_{11}), both of which are also components of the system responsible for the regulation of GS activity by covalent modification, appears to be the accurate assessment of the availability of ammonia (Backman et al., 1983).

TABLE 1.2: GENETICS OF THE gln SYSTEM: DEFINITION OF PHENOTYPES (Magasanik, 1982).

Phenotype	Definition
Gln	Glutamine synthetase
Gln^+	Normal regulation
Gln^-	No enzymatic activity
Gln C	High concentration even in cells grown on a poor carbon source with ammonia and glutamine.
Gln R	Intermediate concentration irrespective of composition of the medium.
Ntr	Enzymes and permeases under nitrogen control
Ntr^+	Increased concentration
Ntr^-	No increased concentration in cells grown on glucose with a poor nitrogen source.
Ntr C	High concentration in cells grown on glucose with an excess of ammonia and glutamine.

TABLE 1.3: THE gln GENES (Magasanik, 1982)

GENE	LOCATION ON *E. coli* CHROMOSOME	PEPTIDE SIZE	PRODUCT	PHENOTYPES OF MUTANTS
gln A	86	55 000	GS	Gln^-, Ntr^-; Gln^-, Ntr C
gln L	86	36 000	pL	Gln C, Ntr C; Gln R, Ntr^-; Gln^+/C, Ntr^+
gln G	86	50 000	pG	Gln R, Ntr^-; Gln^-, Ntr^-
gln F	68	75 000	pF	Gln^-, Ntr^-
gln B	55	11 000	P_{II}	Gln C, Ntr; Gln^- Ntr^-
gln D	4	?	UTase	Gln^-, Ntr^+
gln E	?	?	ATase	Gln^+, Ntr^-

gln E: Mutations in gln E result in the inability of the cell to produce ATase.

gln L: The regulation of GS and of enzymes under nitrogen control in gln L mutants of E. coli is almost normal. The gln L product is not required for the control of gln A expression and other ntr genes, but it apparently communicates redundant information on the availability of ammonia from an ammonia-sensitive system, consisting of the products of the gln B and the gln D genes to the regulatory products of the gln F and gln G genes (Backman et al., 1983).

It was possible to study the expression of gln G in E. coli by fusing this gene to lac Z lacking a promoter. The concentrations of β-galactosidase, GS and histidase were determined in a variety of mutant cells grown under different conditions. The results obtained confirmed that the gln G gene product is both an activator and repressor of transcription initiated at gln Ap, the promoter of gln A, and showed that transcription initiated at gln Ap could proceed through gln G. The transcription initiated at the second promoter gln Lp is subject to repression by the gln G gene product when this product is in the state required for activation of gln Ap. The gln G product, which is an autogenously regulated regulator, is present in high concentration in cells grown under nitrogen limitation, where its transcription is initiated at gln Ap. It is present in low concentrations in cells grown on a poor carbon source with an excess of glutamine and ammonia, where its transcription is initiated

at gln Lp only. The low concentration of the gln G product is sufficient to cause complete repression at gln Ap and when converted to its active form, to activate completely transcription initiated at gln Ap. Activation of hut expression, however, requires a level of gln G product that can be derived solely by gln G transcription initiated at gln Ap when it is activated by the gln G product. High levels of histidase are produced in cells carrying a deletion in the gln ALG region, but which have a functional gln G gene fused to gln Ap on a plasmid. This result indicates that the products of neither gln A nor gln L are required for the activation of hut expression in response to nitrogen starvation. Activation of hut expression therefore depends on the presence of a functional gln F gene.

It may be essential for a cell to be able to initiate transcription of the gln G product from two promoters (Pahel et al., 1982). Repression by the gln G product of transcription initiated at the gln Ap should still allow sufficient transcription of the gln G product from gln Lp to maintain the repression at gln Ap and to allow immediate activation of transcription from gln Ap should the nitrogen level in the medium become growth rate-limiting.

A number of observations have suggested that gln Lp is located between gln A and gln L. Ueno-Nishio et al. (1983) have located a promoter and operator, with the properties of this second regulatory site, at the end of or distal to gln A and includes the site at which translation of gln L is initiated.

Their results suggest that the operator region is located downstream from the promoter.

The gln G gene product regardless of the presence or absence of the gln F product serves as a repressor of transcription initiated at the gln Lp. Results obtained from fusion studies indicate that when transcription of gln L and gln G is initiated at gln Lp all effects of the gln G product can be accounted for by assuming this product to be the specific repressor. Backman et al. (1983) demonstrated that the product of the gln L gene is not essential in the regulation of bacterial nitrogen assimilation. The redundant assessment of ammonia availability by the gln L gene product presumably permits extremely fine control of expression of the regulated gene.

Regulation in response to the nitrogen source thus appears to consist of a central process mediated by the products of the gln F and gln G genes and subjected to modulation by the products of the other genes (Backman et al., 1983).

Many earlier observations can now be explained by this new model. The observation that Mu insertions in gln A (as well as certain point mutations in gln A) result in the Ntr$^-$ phenotype, can be explained by the fact that the level of gln G product , sufficient for activation of genes responsive to ntr can only be achieved by transcription initiated at gln Ap. These point mutations probably affect the polarity

of gln L and gln G with respect to transcription of these genes initiated at gln Ap. This correlates with the fact that the Ntr$^-$ phenotype, but not the Gln$^-$ phenotype, of these mutants can be suppressed by mutations in rho which have resulted in a less efficient transcription termination factor.

Other gln A mutants have the Ntr C phenotype. The level of intracellular P_{II} is presumably insufficient to prevent activation of the gln ALG operon at gln Ap by the gln G and gln F products. The behaviour of gln L mutants can be explained by the fact that the intracellular concentration of the gln L product also depends on which promoter initiates transcription of the gln L gene product.

GENETICS OF NITROGEN REGULATED (ntr) SYSTEMS

If an enzyme or permease is present at a higher concentration in cells grown on glucose with a poor nitrogen source than in cells grown on glucose with ammonia as the nitrogen source, it is subject to nitrogen regulation (Ntr$^+$ phenotype). In addition, mutations in the gln ALG region should affect both GS expression and that of the enzyme or permease being investigated. All mutations in the gln ALG operon, other than deletions in gln L, result in either the Ntr$^-$ or Ntr C phenotypes.

The ntr systems consist of two classes: those enzymes and permeases whose only function is nitrogen assimilation and

those which can also supply the cell with energy and carbon.

The nitrogen-fixation (*nif*) system, which is responsible for the conversion of dinitrogen to ammonia in *K. pneumoniae*, is an example of the first class. The expression of the *nif* genes, in response to fluctuating levels of ammonia, is controlled on two distinct levels (de Bruijn and Ausubel, 1983). The first level is part of the general *ntr* system by which the cell regulates several nitrogen assimilatory genes. The *gln* F and *gln* G gene products activate expression of *gln* A, *nif*, *hut*, *put* and *aut* genes which code for enzymes involved in the degradation of histidine, proline and arginine, respectively. The second level of *nif* regulation involves the specific transcriptional control of the *nif* gene cluster by the *nif* A and *nif* L gene products (Merrick *et al.*, 1982). Under conditions of nitrogen limitation, the *gln* F and *gln* G genes activate the transcription of the *nif* LA transcription unit. The product of the *nif* A gene activates all other *nif* transcription units and this activation requires the *gln* F gene product. The *nif* L gene product serves as a repressor of the *nif* operon in response to rising concentrations of O_2 and NH_4^+. Under conditions of nitrogen excess, the products of the *gln* G and *gln* L genes and possibly the *gln* B gene act together to repress transcription of the *nif* LA transcription unit. A current model for the regulation of the *nif* operon is summarized in Fig. 1.5.(de Bruijn and Ausubel, 1983).

The *nif* L gene product also acts at other *nif* promoters but does not repress its own synthesis (Merrick *et al.*, 1982).

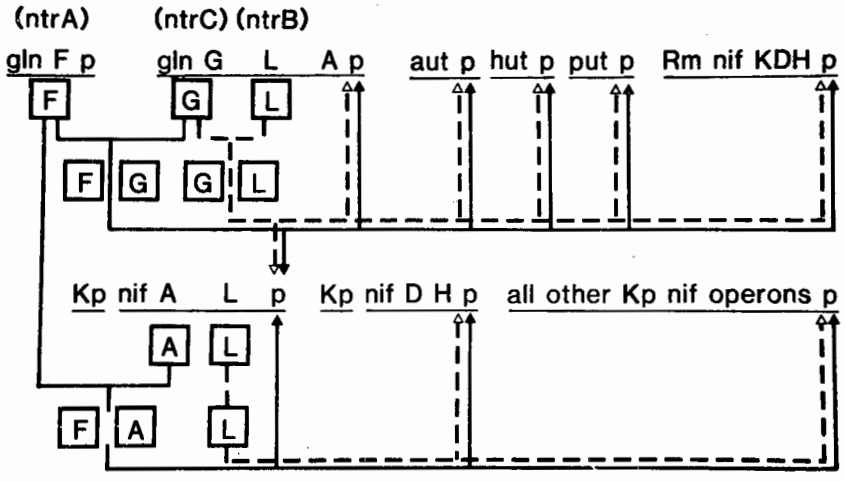


FIG. 1.5: Model for the regulation of ntr controlled genes, such as nif, aut, hut and put. The solid lines with filled-in arrow heads represent pathways of positive regulation. The broken lines with open arrow heads represent pathways of negative regulation. The "p"'s represent promoter-operator regions of the respective transcription units. Kp denotes K. pneumoniae and Rm denotes R. meliloti (de Bruijn and Ausubel, 1983).

Ow *et al.*, (1983) demonstrated that the *K. pneumoniae nif* A gene product can substitute for the *gln* G gene product in activating the promoters of several genes involved in nitrogen assimilation. Tuli *et al.*, (1982) in an elegant study showed that the products of the *gln* A-linked *ntr* genes of *E. coli* can regulate expression of the *hut* and *nif* operons of *K. pneumoniae.*

The model *ntr* system of the second class is the *hut* operon. Studies by Prival and Magasanik (1971) and Rothman *et al.* (1982) have demonstrated that in a wild-type strain starvation for either nitrogen or energy activated the formation of histidase, whereas in a catabolite receptor protein (*crp*) mutant only nitrogen starvation, and in a *gln* G mutant only energy starvation, resulted in this activation of histidase. When the *hut* genes of *K. aerogenes* were transferred to *E. coli*, which lacks this system, histidase was subject to *ntr* and to catabolite repression (Bloom *et al.*, 1977).

Certain enzymes which are capable of supplying the cell with ammonia appear not to be subject to *ntr* in any enteric organism.

GOGAT mutants which are defective in the structural gene for GOGAT (*glt* B), fail to grow on a nitrogen source such as histidine whose degradation provides glutamate. *glt* B mutants are unable to increase their level of GS in response to nitrogen starvation. Presumably, the loss of GOGAT, a glutamine-destroying enzyme makes it impossible for the cell

to lower the intracellular concentration of glutamine sufficiently to activate transcription of gln A. This is supported by the fact that the inability of glt B mutants to grow with glutamate-providing compounds is suppressed by gln L mutations, resulting in the Gln C phenotype. The glt B, gln L double mutants, on the other hand, are unable to use compounds that generate ammonia rather than glutamate, as sources of nitrogen.

In K. aerogenes and K. pneumoniae the formation of GDH is controlled by the gln system. gln G or gln F mutations (Ntr^-) result in high concentrations of GDH, even under conditions of nitrogen starvation. Mutations in gln L (Ntr C) result in a lack of GDH. In K. aerogenes GDH formation seems to be regulated almost opposite to that of the Ntr enzymes. In E. coli and in S. typhimurium, GDH formation is unaffected by gln mutations.

Recent work by Bender et al.,(1983) has shown that the product of a his-linked gene, nac (nitrogen assimilation control), appears to play a major role in nitrogen regulation. It apparently links the synthesis of enzymes that are activated or repressed by ammonia starvation, such as histidase and GDH, to the known regulators of nitrogen assimilation, the products of the gln F and gln G genes. They suggest the possibility that regulation of all systems other than gln ALG requires in addition to the gln F and gln G products, the product of the nac gene. However, they have obtained no

evidence that the mutation observed in nac resulted in the loss of the product of this gene. It is possible that the mutation may have resulted in an altered product that blocks the ability of the gln F and gln G products to regulate expression of the affected genes. This would suggest that the normal product of the nac gene may not play a significant role in the positive and negative control mediated by the gln F and gln G products but may modulate these regulatory effects under certain conditions.

Previous ideas that GS could regulate transcription came from studies on the hut genes of K. aerogenes. The hypothesis suggested that the protein moiety of GS activates expression and was supported by the results of a study that appeared to demonstrate that expression of the hut operon could be activated either by cyclic adenosine 3',5'-monophosphate (cyclic AMP) and the catabolite activator protein (CAP) or by purified deadenylylated GS (Tyler et al., 1974). The view that GS could regulate gene expression was also supported by the observation that mutants with defects in gln A possessed altered Gln and Ntr phenotypes. This was interpreted as evidence that the mutations responsible for Gln C, Ntr C phenotype were in gln A, thus implicating GS as an autogenous regulator. The data did not exclude the possibility that the mutations, resulting in the Gln C phenotype might be contiguous and lie in a neighbouring gene that codes for a regulatory protein and not for the GS polypeptide. Mutations which resulted in the Gln R, Ntr^- phenotypes were closely

linked to gln A mutations (resulting in the Gln$^-$ phenotype) were considered to be alleles of gln A.

In cells of S. typhimurium, GS could not activate hut transcription but it could in the cytoplasm of E. coli or K. aerogenes (Bloom et al., 1977). The authors concluded that GS was necessary for activation of transcription of the hut genes but not sufficient; an additional transcription factor which was missing or altered in S. typhimurium was required. Candidates for this factor included ribonucleic acid polymerase, an enzyme of the GS adenylylation system or the gln F gene product.

Although GS bound to DNA, no sequence specificity was found in the formation of the GS-DNA complex investigated by Streicher and Tyler (1976). Adenylylated GS was thought to repress or be unable to activate gene expression. The addition of ammonia to cells growing under nitrogen limitation caused immediate adenylylation of GS and repressed GS and histidase synthesis. A mutation in the ATase gene of K. aerogenes was reported to have resulted in both the Gln C phenotype and in deadenylylated GS in ammonium-grown cells. However, S. typhimurium mutants lacking ATase were capable of normal regulation of deadenylylated GS. It was subsequently found that the mutation in K. aerogenes resulted in the loss of P_{II} and not ATase. Evidence against GS as an autogenous regulator was also provided by the study of mutations resulting from phage Mu or transposon Tn 10 insertion in the gln A

region of E. coli and S. typhimurium respectively (Pahel and Tyler, 1979; Kustu et al., 1979). Two classes of mutants with Gln^- and Gln R phenotypes resulted and it was shown that the mutations were in separate cistrons, gln A and gln G respectively. This suggested that the product of the gln G gene and not GS was the regulator of gln A expression.

Additional evidence against a regulatory role for GS was provided by studies using an E. coli strain in which the gln A gene had been fused to lac Z, the structural gene for β-galactosidase. Expression of the gln A gene responded to changes in the nitrogen source of the medium, even in the absence of GS. The fusion resulted however in the cell being unable to activate the synthesis of histidase. These findings did not explain why insertions and mutations in gln A affected the Ntr phenotype. The gln ALG operon and the elucidation of the roles of the gln gene products emerged as a breakthrough in the study of the regulation of nitrogen metabolism.

There is little information available on the existence of ntr in bacteria other than the Enterobacteriaceae. A report by Reysset (1981) of a regulatory locus closely linked to gln A in B. subtilis prompts speculation as to whether nitrogen control in organisms other than the enteric coliform bacteria is mediated by a similar genetic system.

Even as the mechanism of catabolite repression is still not fully understood, the intricacies of nitrogen control will probably take considerable time to unravel.

CHAPTER 2

REGULATION OF NITROGEN CATABOLIC ENZYMES IN VIBRIO ALGINOLYTICUS

SUMMARY:

The production of histidase by V. alginolyticus was repressed by a temperature of 37°C and by a lack of oxygen. There was no difference in the growth rate at 30 and 37°C. The inducible nitrogen catabolic enzymes arginase, alanine dehydrogenase and histidase were not repressed by nitrogen catabolite repression. The production of histidase and arginase was stimulated by various amino acids and ammonium ions. Urocanase and formiminoglutamate hydrolase were repressed by nitrogen-containing compounds. The hut enzymes and alanine dehydrogenase were sensitive to catabolite repression by glucose. The addition of $(NH_4)_2SO_4$ markedly enhanced histidase production. Cyclic AMP did not relieve repression by glucose of these levels of histidase production.

2.1. INTRODUCTION:

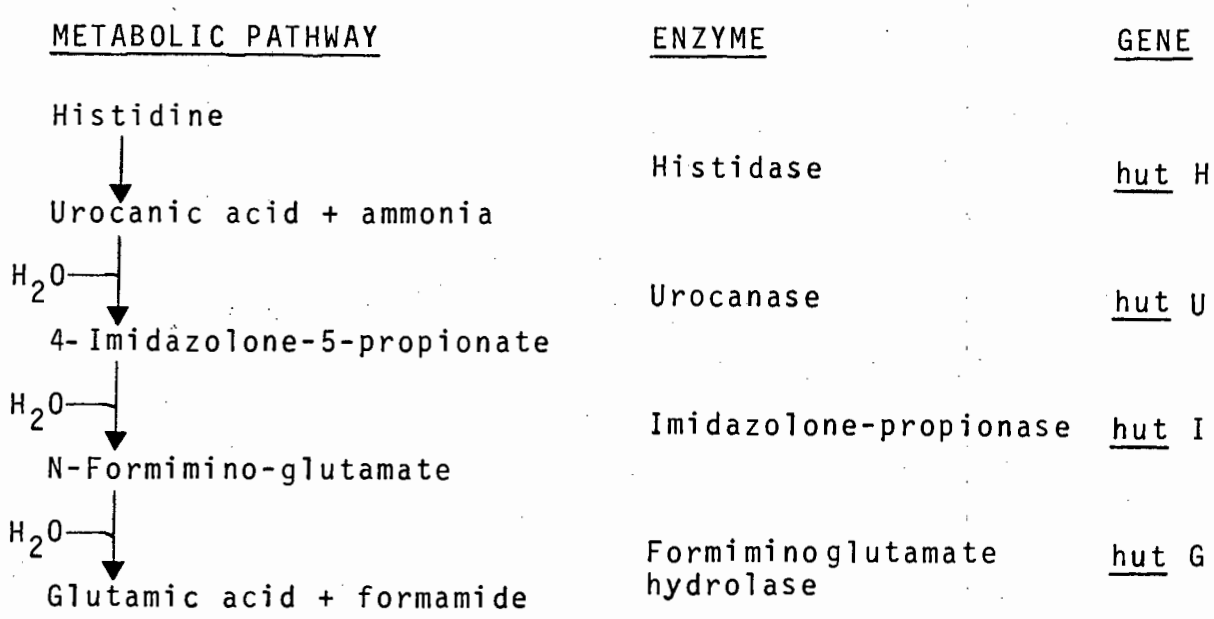
Vibrio alginolyticus is an aerobic, halotolerant, Gram-negative bacterium which produces an extracellular collagenase and extracellular alkaline protease enzymes during the stationary growth phase (Welton and Woods, 1973, 1975; Reid et al., 1978, 1980; Long et al., 1981).

Alkaline protease production is stimulated by histidine and

urocanic acid. Collagenase synthesis, however, is inhibited by these substances (Long et al., 1981). The stimulation of alkaline protease production by histidine and urocanic acid suggests that the histidine utilization (hut) pathway may be involved in the regulation of the alkaline proteases.

In the hut pathway histidine is converted to equimolar amounts of glutamate, ammonia and formamide, as shown in Fig. 2.1.1. The enzymes involved are histidase, urocanase, imidazolone-propionase (IPase) and formiminoglutamate hydrolase (FGA-hydrolase).

FIG. 2.1.1:
The histidine utilization (hut) pathway of S. typhimurium (Smith et al., 1971). The hut pathways in the enteric bacteria and in B. subtilis are identical and are referred to as the enteric-Bacillus pathway (Kendrik and Wheelis, 1982).



Bowden et al. (1982) isolated two groups of V. alginolyticus hut mutants. The first group were histidase hut mutants (hut H) and were characterized by their failure to grow on histidine minimal medium (his-MM), but were able to grow on urocanic acid minimal medium (uro-MM), formimino-L-glutamic acid minimal medium (FGA-MM) and glutamic acid minimal medium (glu-MM). The second group were urocanase mutants (hut U) which were unable to grow on uro-MM but grew on his-MM, FGA-MM and glu-MM. Growth on his-MM was sparse and was presumably due to the utilization of ammonia which was produced with urocanic acid from histidine by histidase. The hut H1 and hut U1 mutants chosen for further study were found to lack histidase and urocanase enzyme activities, respectively. The effect of intermediates in the hut pathway on the production of histidase, urocanase and formimino-hydrolase by V. alginolyticus wild type (WT) cells was investigated in stationary phase cultures (conditions for protease production) and in exponential phase cultures. The hut enzymes were coordinately induced by histidine only. Urocanic acid and formimino-L-glutamic acid did not induce the hut enzymes. In the hut HI mutant, which cannot convert histidine to urocanic acid, histidine was shown to induce the hut enzymes. Although V. alginolyticus is a Gram-negative bacterium, regulation of the hut pathway is not similar to that in S. typhimurium and E. coli. However, it is similar to the Gram-positive Bacillus strains in that histidine and not urocanic acid is the inducer of the hut operon. In the case of enzymes that degrade an essential compound normally produced by the cell, such as histidine, induction by a degradation

product has been considered to be a mechanism that safeguards the cell against a potentially deleterious endogenous induction (Chasin and Magasanik, 1968). The fact that histidine itself induces the histidine-degrading enzymes in V. alginolyticus and B. subtilis indicates that these organisms can exist without this safeguard, or that they possess another mechanism to prevent endogenous induction.

Although the hut enzymes were induced by histidine, the induction of alkaline protease synthesis in V. alginolyticus was stimulated by urocanic acid. Histidine is an inducer by virtue of its conversion to urocanic acid. Studies with the hut H1 and hut U1 mutants confirmed these suggestions, since alkaline protease production in the hut H1 mutant was stimulated by urocanic acid and not by histidine. Not surprisingly, alkaline protease activity in the hut U1 mutant was stimulated by both histidine and urocanic acid. The decreased constitutive level of alkaline protease synthesis in the hut U1 mutant in MM is interesting since elevated constitutive protease synthesis would normally have been expected. Urocanic acid would be expected to accumulate in the hut U1 mutant, since histidine made endogenously would give rise to urocanic acid, which cannot be metabolized, and would therefore induce alkaline protease synthesis. This suggests that the regulation of alkaline protease synthesis by the hut operon is complex, and does not only involve urocanic acid concentrations. Coregulation of the hut operon and collagenase is suggested by inhibition of

collagenase synthesis in the hut mutants (Bowden et al., 1982).

Many enzymes are subject to control by end-product and catabolite repression. A widely reported example of the former is the repression of enzyme synthesis by amino acids, which has been reported in many bacterial genera, including the Bacillus spp. (May and Elliott, 1968). In addition to end-product repression, some enzymes are subject to catabolite repression by various carbon sources. Catabolite repression is the permanent repression of inducible or constitutive enzyme synthesis that occurs in the presence of glucose or some other rapidly metabolized carbon source (Priest, 1977). Cyclic adenosine 3',5'-monophosphate (Cyclic AMP) at low concentration relieves catabolite repression by glucose of the synthesis of many inducible enzymes in E. coli. It has been proposed that since glucose lowers the intracellular concentration of cyclic AMP, the intracellular level of cyclic AMP regulates the rate of synthesis of many inducible enzymes in E. coli and other microorganisms (De Crombrugghe et al., 1969).

Environmental factors other than individual nutrients in the environment such as temperature and oxygen also play a role in enzyme regulation but have not been widely investigated. The expression of the nitrogen fixation (nif) operon in the facultative anaerobe Klebsiella pneumoniae is sensitive to repression by NH_4^+ (Eady et al., 1978), oxygen (St John et al.,

1974; Eady et al., 1978) and temperature (Hennecke and Shanmugan, 1979). The hut operon in this organism is regulated by NH_4^+ and cyclic AMP (Prival et al., 1973), and by oxygen (Goldberg and Hanau, 1980). Studies on the regulation of nitrogenase synthesis by temperature (Hennecke and Shanmugan, 1979) and oxygen (Hill et al., 1981) have implicated the synthesis of specific proteins which appear to have a regulatory role. Hare et al. (1981) showed that the production of the extracellular collagenase and the alkaline protease by V. alginolyticus was affected by a temperature shift from 30 to 37°C and by a lack of oxygen.

Control of the synthesis of the enzyme systems responsible for utilization of various nitrogen-containing compounds as a function of the nitrogen source used for growth has been termed nitrogen catabolite repression (Tyler, 1978). The phenomenon of nitrogen catabolite repression has been shown to be a control mechanism in enteric organisms such as E. coli, K. aerogenes, and S. typhimurium and in yeasts and fungi. In contrast, the inducible catabolic enzymes arginase and alanine dehydrogenase from B. licheniformis and histidase from B. subtilis are not regulated by nitrogen catabolite repression (Schreier et al., 1982).

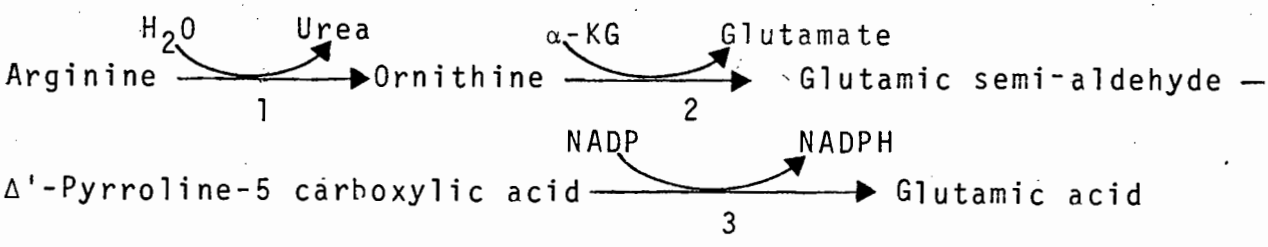
Although V. alginolyticus is a Gram-negative bacterium it is similar to Gram-positive Bacillus strains in a number of respects: they produce true extracellular proteases during the stationary growth phase (Welton and Woods, 1973; Welton

and Woods, 1975; Priest, 1977; Long et al., 1981); protease production is rifampin-insensitive (Both et al., 1972; O'Connor et al., 1978; Reid et al., 1980) and is subject to end-product and catabolite repression which is not relieved by cyclic AMP (Priest, 1977; Long et al., 1981; Glenn, 1976; Reid et al., 1978). In B. subtilis and V. alginolyticus histidine and not urocanic acid is the inducer of the hut enzymes (Chasin and Magasanik, 1968; Bowden et al., 1982). Since aspects of enzyme regulation in V. alginolyticus resemble Bacillus strains rather than the more closely related Gram-negative strains, the phenomenon of nitrogen catabolite repression in V. alginolyticus was investigated.

L-alanine dehydrogenase is a key enzyme in the catabolism of L-alanine to pyruvate and may be required for further catabolism of alanine by the tricarboxylic acid cycle (McCowen and Phibbs, 1974).

Arginine is known to serve as a sole nitrogen source by virtue of its catabolism in three steps to glutamate. The first enzyme in the pathway, arginase, catalyzes the cleavage of arginine to ornithine and urea (Fig. 2.1.2).

FIG. 2.1.2. PATHWAY OF ARGININE CATABOLISM IN B. LICHENIFORMIS (BROMAN ET AL., 1978)



1: Arginase 2: Ornithine transaminase 3: Pyrroline dehydrogenase

2.2. MATERIALS AND METHODS

All % values are w/v unless otherwise stated.

2.2.1. BACTERIAL STRAINS AND MEDIA

Media are listed in the Appendix. The collagenolytic strain previously isolated and classified as *Achromobacter iophagus* by Welton and Woods (1973) but recently classified as a *V. alginolyticus* strain was used (Reid *et al.*, 1980). The *hut* H1 mutant isolated by Bowden *et al.* (1982) was also used.

2.2.2. MAINTENANCE OF V. ALGINOLYTICUS

For the long term maintenance of *V. alginolyticus* the strain was kept at 20°C in 0.1 M Tris-HCl buffer (pH 7.6) containing approximately 5 mg ml^{-1} of bovine Achilles' tendon collagen. For routine use the culture was maintained on ammonia succinate minimal medium (ASMM) and on the complex medium of Welton and Woods (1973).

2.2.3. GROWTH CONDITIONS FOR ENZYME STUDIES

To facilitate aeration, loosely fitting aluminium foil caps were used and culture volumes were 5 to 10% of the flask volume (Reid, 1981).

Samples (10 ml) of overnight *V. alginolyticus* cultures in ASMM were harvested by centrifugation, washed twice in 0.1 M Tris-HCl buffer (pH 7.6) and resuspended in SMM supplemented with a particular nitrogen source. In the experiments where a particular nitrogen-containing compound served as the sole

nitrogen source, $(NH_4)_2SO_4$ was omitted from the ASMM. The cultures were aerated on a Gallenkamp orbital shaker at 140 rev. min^{-1} at 30°C. Growth was followed turbidimetrically at 600 nm using an MSE Spectro - plus spectrophotometer. Mid-exponential samples were harvested by centrifugation, washed twice in 0.05 M K_2PO_4 buffer (pH 7.6) or 50 mM Tris-HCl + 200 mM KCl (pH 9.0) when arginase activity was being assayed. The pellets were resuspended (at 1.25% of the original sample volume) in the phosphate buffer; (10 mM Tris-HCl, pH 9.0 was used for the arginase sample). The cells were frozen in liquid nitrogen and the thawed suspensions were disrupted by sonic treatment (6 times for 30 s with 30 s intervals for cooling) with an MSE-ultrasonic disintegrator set at 25 kHz. Cell debris was removed by centrifugation at 16,000 rpm for 40 min at 4°C. Enzyme activities of the cell extracts were determined using the assay procedures outlined below. All enzyme assays were performed at standardized cell densities. Each sample was assayed in duplicate and experiments were repeated at least four times.

2.2.4. HISTIDASE ASSAY

Samples (2 ml) of exponential cultures were added to 0.5 ml cold toluene and 0.1 ml samples were assayed for histidase according to the methods of Hartwell and Magasanik (1963) and Chasin and Magasanik (1968). The samples were added to the reaction mixture containing 0.1 ml of 1 M diethanolamine-HCl (pH 9.4), 0.2 ml of 0.1 M L-histidine, 0.2 ml of H_2O and 0.4 ml of 0.05 M K_2PO_4 buffer (pH 7.4), which was maintained

at 37°C. The enzyme reaction was allowed to proceed for 30 min at 37°C.

The reaction was stopped by the addition of a saturated solution of sodium tetrahydroborate. A blank lacking histidine was included for each extract tested. The production of urocanic acid was measured at an absorbance of 277 nm on an MSE-Spectroplus spectrophotometer. One unit of histidase activity is defined as the amount of enzyme that results in an increase in absorbance of 0.1 at 277 nm in 30 min at 37°C (Bowden et al., 1982).

2.2.5. UROCANASE ASSAY

Unlike histidase, neither urocanase nor FGA-hydrolase could be assayed in toluenized cells as a result of the low activity of the former and the low sensitivity of the assay for the latter. These enzymes were assayed in sonic extracts of cells. Samples (0.1 ml) were added to the reaction mixture containing 0.4 ml of 0.5 M K_2PO_4 buffer (pH 7.4), 0.02 ml of 0.4 M EDTA, 1.28 ml of H_2O and 0.2 ml of 0.8 mM urocanic acid, which was maintained at 37°C. The enzyme reaction was allowed to proceed for 60 min at 37°C. Urocanic acid degradation was measured as the difference in absorbance at 277 nm before and after incubation at 37°C. It was also necessary to include a blank lacking urocanic acid to compensate for slight increases in optical density which sometimes occurred in the absence of this substrate. One unit of urocanase activity is defined as the amount of enzyme that results in

a decrease in absorbance of 0.1 at 277 nm in 60 min at 37°C.

2.2.6. FGA-HYDROLASE ASSAY

FGA-hydrolase activity was assayed according to the methods of Lund and Magasanik (1965) and Chasin and Magasanik (1968). The FGA was stored as the barium salt. For use in enzymatic experiments, it was suspended in H_2O and the barium was removed by the addition of an equivalent amount of Na_2SO_4.

Samples (0.1 ml) were added to a reaction mixture containing 0.04 ml of 1 M Tris-HCl (pH 9.0), 0.02 ml of 0.1 mM $MnCl_2$ and 0.02 ml of 0.1 M glutathione, which was maintained at 37°C. The enzyme reaction, initiated by the addition of 0.12 ml of a solution containing 1 µmol of FGA, was allowed to proceed for 15 min at 37°C. The reaction was terminated by the addition of 1.6 ml of a saturated sodium borate solution. The FGA remaining was assayed by the addition of 0.4 ml of colour reagent (4 g of NaOH, 4 g of sodium nitroprusside, and 4 g of potassium ferricyanide in 120 ml of water) to each tube. The resulting colour was read after 30 min at 15°C at 485 nm in a DU-8 spectrophotometer. Controls involved reaction mixtures to which borate was added before FGA. One unit of FGA-hydrolase activity is defined as the amount of enzyme that results in a decrease in absorbance at 485 nm of 0.1 in 15 min at 37°C.

2.2.7. ALANINE DEHYDROGENASE ASSAY

Alanine dehydrogenase was assayed according to the method of McCowen and Phibbs (1974), but with modifications. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol and 70 mM L-alanine in a total volume of 3.0 ml. Controls involved reaction mixtures lacking alanine. Sonic extract (0.15 ml) was added to 0.15 ml of reaction mixture. maintained at 22°C. The reaction was initiated by the addition of nicotinamide adenine dinucleotide (NAD) to a final concentration of 2 mM. The reaction was allowed to proceed for 2.5 min at 22°C. The appearance of reduced NAD (NADH) was measured at an absorbance of 340 nm on a DU-8 spectrophotometer. One unit of alanine dehydrogenase activity is defined as the amount of enzyme that results in an increase in absorbance of 0.1 at 340 nm in 2.5 min at 22°C.

2.2.8. ARGINASE ASSAY

Arginase was assayed according to the method of Ratner (1962). The assay is based on the estimation of ornithine production by a colorimetric procedure. The reaction reads as follows: L-arginine + L-glycine $\rightleftharpoons$ guanidoacetate + L-ornithine. Samples (0.1 ml) were added to the reaction mixture containing 0.15 ml of 0.1 M L-arginine, 0.25 ml of 0.1 M L-glycine and 1.0 ml of 1.0 M K_2PO_4 (pH 7.5), which was maintained at 37°C. The reaction was allowed to proceed for 20 min at 37°C. The reaction was stopped with 2 ml of 8.3% trichloroacetic acid (TCA). Controls containing TCA which was added before glycine

were used to measure endogenous activity present in the extracts. The colour reagent for the determination of ornithine contained 250 mg ninhydrin and 37.6 mg hydrindantin dissolved in a mixture of 4 ml of 6 M H_3PO_4 and 6 ml of glacial acetic acid, by boiling briefly. Samples (0.5 ml) of the TCA filtrate were added to 0.5 ml of H_2O, 1.0 ml of the warm colour reagent and 1.5 ml of glacial acetic acid. This mixture was heated for 30 min at 100°C and then placed in H_2O for 10 min at 25°C. Glacial acetic acid was added to a final volume of 6 ml. The production of ornithine was measured at an absorbance of 515 nm.

One unit of arginase activity is defined as the amount of enzyme that results in an increase in absorbance of 0.1 at 515 nm in 20 min at 37°C.

2.3. RESULTS

2.3.1. INDUCTION OF HISTIDASE BY HISTIDINE

The *V. alginolyticus* W.T. strain and the *hut* H1 mutant were grown on uro-SMM, uro + ASMM and his + ASMM and their histidase activities were determined during the growth cycle (Fig. 2.3.1). The W.T. strain grown in his + ASMM showed induction of histidase. Urocanic acid did not induce the enzyme. The *hut* H1 mutant definitely lacked histidase activity.

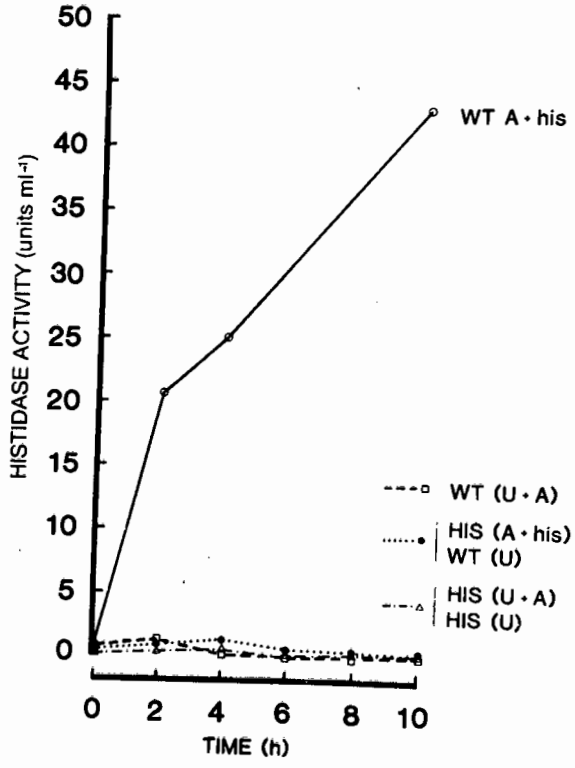


FIG. 2.3.1: Induction of histidase by V. alginolyticus W.T. and the hut H1 mutant strain in uro-SMM (●; △), uro + ASMM (□ ; △) and his + ASMM (○ ; ●) respectively. Each point is calculated as the average of four independent determinations.

2.3.2. EFFECT OF TEMPERATURE AND OXYGEN ON GROWTH AND HISTIDASE PRODUCTION

The effects of temperature and oxygen on growth and on histidase levels of the W.T. strain were investigated. The growth rates of V. alginolyticus cultures at 30 and 37°C were very similar (Fig. 2.3.2). The growth rate of exponential phase cultures was affected by aeration. Aerated cultures grew faster than standing cultures (Fig. 2.3.2). The highest levels of histidase activity were produced by V. alginolyticus cells in aerated cultures at 30°C (Fig. 2.3.3). A lower level of histidase activity was produced at 37°C. In non-aerated cultures at 30°C markedly lower levels of histidase were observed (Fig. 2.3.3). Standing cultures resulted in a 67% reduction in histidase activity.

2.3.3. EFFECT OF AMMONIUM IONS AND GLUCOSE ON HUT ENZYME LEVELS IN V. ALGINOLYTICUS

The addition of increasing concentrations of ammonium ions to growing cultures did not repress the production of histidase but enhanced its production (Table 2.3.1). The highest level of histidase activity was obtained with 500 mM $(NH_4)_2SO_4$. The opposite effect was obtained with urocanase and FGA-hydrolase which were both repressed by the addition of 0.5 mM $(NH_4)_2SO_4$. The addition of NH_4^+ to enzyme extracts prepared from his-SMM cultures did not markedly affect the activity of cell-free histidase and urocanase (Table 2.3.2) compared with enzyme levels demonstrated during induction or repression (Table 2.3.1).

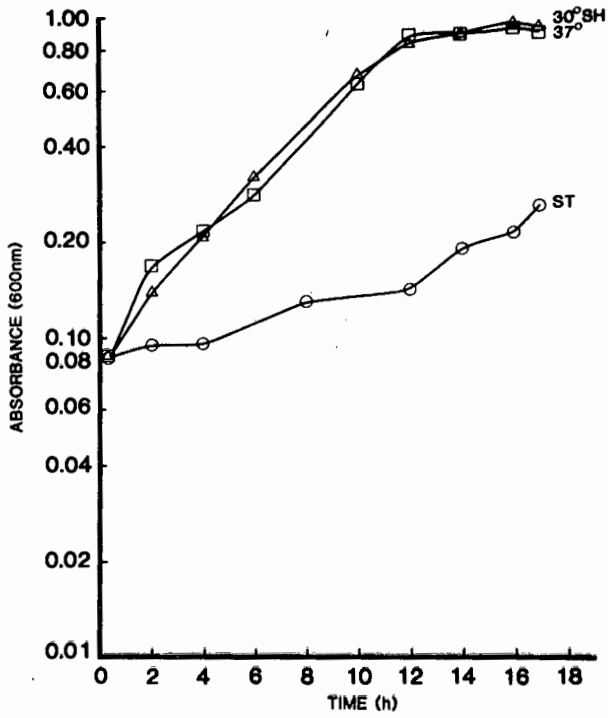


FIG. 2.3.2: Effect of temperature and oxygen on the growth of V. alginolyticus in his-SMM. Cultures incubated at 30°C with aeration (△), 37°C with aeration (□) and at 30°C without aeration (○).

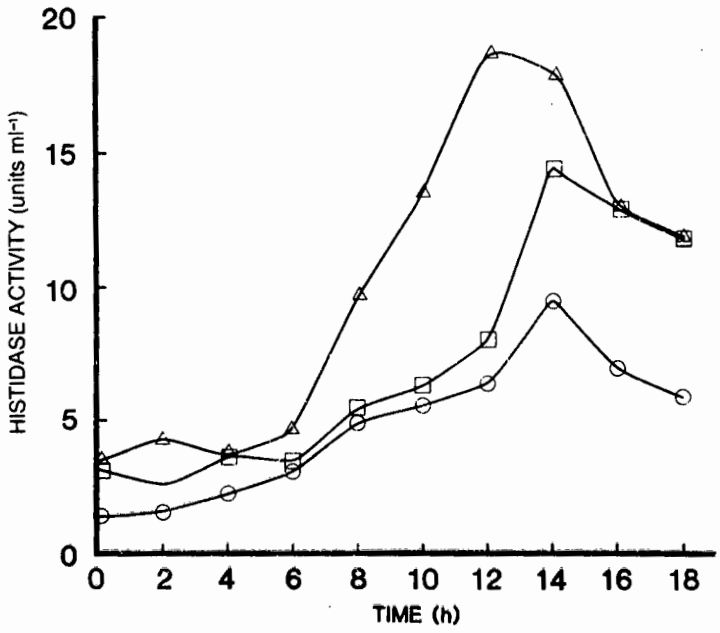


FIG. 2.3.3: Effect of temperature and oxygen on histidase production by V. alginolyticus. Cultures incubated at 30°C with aeration (△), 37°C with aeration (□) and at 30°C without aeration (○). Each point is calculated as the average of four different determinations.

TABLE 2.3.1. Effect of ammonium ions and glucose on *hut* enzyme levels in *V. alginolyticus*. Cultures were assayed at standardised cell densities for histidase, urocanase and formimino-hydrolase enzyme activities 4 h after resuspension in ASMM and his-SMM. *hut* enzyme activities were expressed as a percentage of the activity in his-SMM.

Medium	$(NH_4)_2SO_4$ (mM)	Glucose (mM)	Enzyme activity (%)		
			Histidase	Urocanase	Formimino-hydrolase
ASMM	7.5	0	40	10	34
His-SMM	0	0	100	100	100
His-SMM	0.5	0	169	33	27
His-SMM	10.0	0	176	29	28
His-SMM	500.0	0	195		
His-SMM	0	22	51	29	33

TABLE 2.3.2. Effect of ammonium ions on the activities of cell-free histidase and urocanase. Enzyme activities were expressed as a percentage of the activity with no addition of NH_4^+.

$(NH_4)_2SO_4$ (mM)	Enzyme activity %	
	Histidase	Urocanase
0	100	100
0.5	105	108
10	105	110
100	98	90
500	91	90

The effect of glucose (22 mM) on hut enzyme levels was investigated. Glucose repressed the production of histidase, urocanase and FGA-hydrolase in his-SMM (Table 2.3.1). The addition of cyclic AMP (5 mM) did not relieve the repression of histidase by glucose, but it had the reverse effect, and it repressed histidase production even further (Table 2.3.3).

2.3.4. EFFECT OF AMINO ACIDS AND AMMONIUM IONS ON THE PRODUCTION OF HISTIDASE AND UROCANASE

The effects of the addition of amino acids and ammonium ions on histidase and urocanase production were investigated. The amino acids, arginine, alanine, proline, leucine, glycine and glutamic acid all stimulated the production of histidase but repressed or did not markedly affect the production of urocanase (Table 2.3.4). Tryptophan, glutamine and isoleucine repressed the production of histidase and urocanase to various levels. Ammonium ions stimulated histidase activity but repressed urocanase activity, which was consistent with previous results.

2.3.5. INDUCTION AND EFFECT OF NITROGEN SOURCE ON ALANINE DEHYDROGENASE PRODUCTION

Alanine dehydrogenase was only produced in the presence of alanine and induction of alanine dehydrogenase by alanine was not repressed by $(NH_4)_2SO_4$, urea and nitrate (Table 2.3.5). Slightly lower levels of alanine dehydrogenase were produced in basal media containing glutamic acid or glutamine.

TABLE 2.3.3. Effect of glucose (11 mM) and cyclic AMP (5 mM) on the ammonia-enhanced histidase activity in V. alginolyticus. Cultures were assayed at standardized cell densities for histidase activity 4 h and 8 h after resuspension in his-SMM supplemented with 0.5 mM $(NH_4)_2SO_4$. Histidase activity was expressed as a percentage of the activity in 0.5 mM $(NH_4)_2SO_4$ + his-SMM.

Medium	$(NH_4)_2SO_4$ (mM)	Glucose (mM)	cyclic AMP (mM)	Histidase activity (%) 4 h	8 h
His-SMM	0.5	0	0	100	100
His-SMM	0.5	11	0	78	73
His-SMM	0.5	11	5	61	58

TABLE 2.3.4. Effect of amino acids and ammonium ions on the production of histidase and urocanase. Amino acids were added at 0.5% (w/v). Additions were made at the time of resuspension in his-SMM and enzyme levels assayed at standardized cell densities 4 h after resuspension. Enzyme activities were expressed as a percentage of the activity in his-SMM.

Addition	Concentration (mM)	Histidase activity (%)	Urocanase activity (%)
None		100	100
$(NH_4)_2SO_4$	38	170	16
L-Arginine	24	403	41
L-Alanine	56	222	38
L-Proline	43	182	85
L-Leucine	38	164	42
L-Glycine	67	150	105
L-Glutamic acid	34	140	106
L-Tryptophan	25	88	86
L-Glutamine	34	67	24
L-Isoleucine	38	65	78

TABLE 2.3.5. Effect of nitrogen source on alanine dehydrogenase production in the presence and absence of alanine. Cultures were assayed at standardized cell densities 4 h after resuspension in SMM containing various nitrogen sources (20 mM) and in the absence (basal) and presence of alanine (20 mM).

Nitrogen Source	Alanine dehydrogenase activity (units ml^{-1})	
	Basal	Alanine
Alanine		145
$(NH_4)_2SO_4$	18	147
Urea	5	158
KNO_3	13	149
L-Glutamic acid	13	114
L-Glutamine	28	116

TABLE 2.3.6. Effect of ammonium ions on arginase levels in *V. alginolyticus*. Cultures were assayed at standardized cell densities 4 h after resuspension in ASMM and arg-SMM (24 mM arginine) containing various concentrations of $(NH_4)_2SO_4$.

Medium	$(NH_4)_2SO_4$ (mM)	Arginase (units ml^{-1})
ASMM	0	0.30
Arg-SMM	0	1.98
Arg-SMM	0.5	8.95
Arg-SMM	10	3.71
Arg-SMM	100	2.23

2.3.6. INDUCTION AND EFFECT OF AMMONIUM IONS ON ARGINASE PRODUCTION

Arginase production was induced by arginine. The addition of varying concentrations of ammonium ions enhanced the production of arginase (Table 2.3.6).

2.4. DISCUSSION

Although *V. alginolyticus* is a Gram-negative bacterium, regulation of the *hut* pathway is similar to that in the Gram-positive *B. subtilis*: histidine, rather than urocanic acid, is the actual inducer. The results reported in this thesis confirm the results of Bowden *et al.* (1982).

Histidase production was repressed by incubation at 37°C and a lack of oxygen. Hare *et al.* (1981) showed that collagenase production was totally inhibited by 37°C and that alkaline protease production was markedly reduced. Standing cultures showed markedly decreased levels of exoenzyme synthesis. Growth rates of *V. alginolyticus* were identical at 30 and 37°C but cultures required good aeration for optimal growth. Control of enzyme production by temperature seems to be specific as growth and macromolecular synthesis are not affected (Hare *et al.*, 1981).

Induction of histidase occurred even though ammonia, a readily metabolizable nitrogen source, was present in the medium (Fig. 2.3.1). The control system involving nitrogen catabolite

repression allows microorganisms growing on 'preferred' nitrogen sources to bypass the synthesis of unneeded nitrogen catabolic enzymes. These enzymes are induced when the preferred nitrogen sources are not available and growth of the organism is slow. Preferred nitrogen sources are rapidly metabolizable and yield high growth rates (Schreier *et al.*, 1982). In the enteric organisms, growth in the presence of ammonia (a preferred compound) and histidine (a non-preferred compound) as nitrogen source leads to rapid growth (ammonia utilization) and the repression of the *hut* enzymes (Tyler, 1978). This contrasts with the situation in *V. alginolyticus* which resembles *Bacillus* spp. where the levels of inducible nitrogen catabolic enzymes are not subject to nitrogen catabolite repression (Schreier *et al.*, 1982).

In *V. alginolyticus* histidase and arginase were not subject to control by end-product repression. Various amino acids and ammonium ions did not repress enzyme production but in some cases actually stimulated it. The mechanism of this control is unknown. Cell-free enzyme activities were not affected by ammonium ions and the effect of ammonia is therefore not a catalytic one, affecting enzyme activity during a reaction, but a growth-induced phenomenon.

Although the *hut* enzyme histidase was not regulated by nitrogen catabolite repression, urocanase and formimino-hydrolase were repressed by nitrogen-containing compounds. This suggests that in *V. alginolyticus* histidase is regulated independently

of the other hut enzmes and is situated in a different operon. However, all the hut enzymes are coordinately induced by histidine. The production of imidazolone-propionase, which is the third enzyme in the hut pathway was not investigated because of difficulties in procuring and storing 4-imidazolone-5-propionate (Smith et al., 1971).

The addition of exogenous cAMP did not relieve repression of histidine by glucose, in fact it repressed histidase activity even further. The failure of cyclic AMP to overcome the glucose effect has been reported for enzyme synthesis in Vibrio parahaemolyticus (Tanaka and Iuchi, 1971), Pseudomonas maltophilia (Boethling, 1975), Pseudomonas lemoignei (Stinson and Merrick, 1974), Staphylococcus aureus (Yoshikawa et al., 1974) and many Bacillus spp. (Priest, 1977). Cyclic AMP actually inhibited the production of both the protease and collagenase activities in V. alginolyticus (Reid et al., 1978). Priest (1977) has reviewed the role of cyclic AMP in the genus Bacillus and concludes that it is not the universal regulator molecule that it was once thought to be as it cannot be detected in several bacilli.

The addition of ammonium ions enhanced histidase and arginase synthesis, repressed urocanase and FGA-hydrolase activities and did not affect alanine dehydrogenase levels. Ammonium ion addition repressed collagenase and protease production. Although histidase and protease production were both increased by histidine, the production of protease enzymes was repressed by ammonium ions. Histidine repressed collagenase production.

It would seem that collagenase and protease are situated on different operons as they are not coordinately induced or repressed. It is not yet clear what the involvement of *hut* is in the regulation of protease and collagenase. Further work involving the use of mutants will yield interesting information. Mutants which have overcome control of collagenase and protease by temperature and oxygen and which do not show histidase enhancement on $(NH_4)_2SO_4$ addition would help in determining the exact nature of the regulatory mechanisms involved.

The data presented here do not exclude transport phenomena from a role in repression or enhancement; for instance, addition of amino acids or ammonium ions to a his-SMM grown culture could result in diminished or increased histidine uptake. Since the level of histidase remains enhanced or repressed over the whole growth cycle, however, suggests that this is not the case.

Genetic analysis of *V. alginolyticus* has been attempted (Reid, 1981). However, attempts at transformation and in isolating bacteriophages specific for *V. alginolyticus* proved unsuccessful. As an alternative to developing an *in vitro* cell-free translation system, the techniques for a *V. alginolyticus* gene bank in *E. coli* have been developed and if successful will prove invaluable in determining whether regulation of these systems is affected or altered in another Gram-negative organism.

CHAPTER 3

REGULATION OF THE AMMONIA ASSIMILATORY ENZYMES IN V. ALGINOLYTICUS

SUMMARY

Regulation of the ammonia assimilatory enzymes and particularly GS in V. alginolyticus was studied. GS activity was assayed according to the γ-glutamyl transferase reaction (GS_T activity) and the Mg^{2+}- and Mn^{2+}-dependent biosynthetic reactions (GS_B activity). The forward transferase reaction and inhibition of the transferase activity by 60 mM Mg^{2+} was not demonstrated for V. alginolyticus GS. An NH_4^+-induced loss of GS_T activity occurred in V. alginolyticus cells. Both NH_4^+-shocked and unshocked cells exhibited a pH optimum at 7.9. No isoactivity point could be defined. Although an adenylylation system does exist in V. alginolyticus (P. Brandt, personal communication), the decrease in GS_T activity produced on addition of $(NH_4)_2SO_4$ is primarily due to some hitherto unknown mechanism or to direct feedback inhibition by $(NH_4)_2SO_4$.

GS inactivation in crude extracts by $(NH_4)_2SO_4$ addition was not accompanied by an increase in Mn^{2+}-dependent GS_B activity. The Mg_2^+-dependent GS_B activity was higher for both shocked and unshocked cells. Dialysis for 18 h did not affect the GS_T and GS_B activities of crude extracts of shocked and unshocked cultures.

GS_T activity in cell-free extracts was feedback ihibited by AMP, $(NH_4)_2SO_4$, tryptophan, arginine and glycine. The combined effect of some amino acids was antagonistic. GS_T activity was derepressed when glutamate was the sole nitrogen source for growth. However, it was repressed when growth was supported by an equal concentration of $(NH_4)_2SO_4$ or when both glutamate and $(NH_4)_2SO_4$ were simultaneously present in the medium. Both GS_T and GS_B activities were enhanced by growth in various amino acid-containing media, especially pro-SMM. Growth with glutamine as sole nitrogen source severely repressed GS_T activity.

Growth of V. alginolyticus in the presence of glucose, sucrose, fructose, glycerol and maltose stimulated GS_T activity. Arabinose and lactose, however, both repressed GS_T activity.

GS in V. alginolyticus was subject to regulation by repression and derepression as a complex function of both the carbon and nitrogen source, feedback inhibition, response to divalent cation concentration and buffer, and by an adenylylation-deadenylylation system.

GOGAT production was repressed under conditions of nitrogen excess and derepressed when nitrogen was growth rate-limiting. Thus, a reciprocal relationship existed between GOGAT and GS

when $(NH_4)_2SO_4$ was the sole nitrogen source. The opposite situation was observed with GDH. Synthesis of GDH was markedly increased when the $(NH_4)_2SO_4$ concentration was high and its production was repressed when $(NH_4)_2SO_4$ was in short supply. When glutamate was used as sole nitrogen source, the production of both GOGAT and GDH was low. Glucose repressed the production of GOGAT and did not stimulate the level of GDH in a nitrogen-limited medium.

The major route of ammonia assimilation in V. alginolyticus under conditions of nitrogen limitation was the coupled GS-GOGAT pathway, while under conditions of nitrogen excess, the GDH pathway was operative.

3.1. INTRODUCTION

The two alternate routes for ammonia assimilation in micro-organisms were discussed in Section 1.1. The GS-GOGAT pathway is physiologically irreversible and has a high affinity of GS for ammonia. GDH catalyzes a reversible reaction and has a low affinity for ammonia. This is in accord with their roles at low and high concentrations of ammonia respectively. In S. typhimurium GDH, which functions primarily at high ammonia levels, does not decrease in cells grown in the presence of a nitrogen-limiting source (Brenchley et al., 1975). This is in contrast to the situation in K. aerogenes (Dendinger et al., 1980).

The amino acids glutamate and glutamine are used as amino and amido donors for other nitrogenous compounds as well as for

protein synthesis (Dendinger et al., 1980). Thus the regulation of the three enzymes that synthesise glutamate and glutamine may have some novel features in addition to control elements common to other biosynthetic pathways. Much of the physiological and genetic work examining the regulation of nitrogen utilization has centred on the glutamine biosynthetic enzyme, GS, rather than on the glutamate biosynthetic enzyme, GOGAT. The ubiquitous distribution of the GS-GOGAT pathway of ammonia assimilation indicates its fundamental role in the growth of organisms in natural ecosystems (Tempest et al., 1973).

Since the ammonia assimilatory enzymes (particularly GS) were shown to be involved in the nitrogen catabolite repression control mechanism in enteric bacteria (Tyler, 1978), these enzymes in V. alginolyticus under various cultural conditions were investigated.

Perhaps the simplest screening technique for the presence of an adenylylation-deadenylylation system in bacteria, is to test for the rapid inactivation of GS on the addition of $(NH_4)_2SO_4$ to cells growing in glutamate medium (Wohlhueter et al., 1973). It was in fact this phenomenon that led to the discovery of the interconversion of GS. However, crucial to the interpretation of this inactivation as a chemical interconversion was the fact that the GS_T activity was relatively unaltered by this treatment. Adenylylation allows for a rapid readjustment of nitrogen metabolism in response to

changes in the nature of the exogenous source of nitrogen. In cells growing in a glutamate medium, ammonium is the limiting factor for glutamine synthesis; the added ammonium causes a burst of glutamine synthesis imposing a drain on ATP. The accumulated glutamine activates the ATase, which in turn inactivates GS and thus allows repletion of the ATP pool.

The GS of V. alginolyticus has recently been purified and was found to be atypical regarding its size and sedimentation coefficient ($S_{20,W}$) (Brandt, 1983). Studies with the dissociated enzyme suggest that the subunit MW is approximately 62 000. If the enzyme is a typical dodecamer, then its MW is estimated to be approximately 744 000. The $S_{20,W}$ of the GS of V. alginolyticus was 23.9. The GS from E. coli has a MW of 600 000 and a $S_{20,W}$ of 19.35 (Tyler, 1978). Electron micrographs indicate that the GS of V. alginolyticus has a hexagon-like structure with a central hole. The GSs of Rhizobium japonicum strains CC705 and CC723 have a MW of 720 000 and consist of 12 subunits of 60 000 (Bhandari et al., 1983).

3.2. METHODS

3.2.1. ENZYME PREPARATIONS FOR GS ASSAYS

Sonic extracts were prepared as described in 2.2, with the following modification. The cells were washed in 1% KCl buffer and resuspended in 10 mM imidazole buffer (pH 7.15) containing 2.5 mM $MgCl_2$.

For whole cell preparations, growth was stopped and the adenylylation state fixed by the addition of hexadexyltrimethylammonium bromide (CTAB; 1 mg/ml) (Bender et al., 1977). Aeration of the cultures was continued for 5 min at 37°C. The cells were harvested by centrifugation at 4°C, washed once with 1% KCl and resuspended in 1% KCl. Extracts were prepared from bacteria harvested in the mid-exponential growth phase. All enzyme assays were performed at standardized cell densities. Each sample was assayed in duplicate and experiments were repeated at least three times.

3.2.2. "NH_4^+-SHOCKING" PROCEDURE

A culture of V. alginolyticus was grown on 20 mM L-glutamic acid SMM (glu-SMM) to mid-exponential growth phase. One half of this culture was harvested and the other half was "shocked" by the addition of 15 mM $(NH_4)_2SO_4$ and harvested 7 min later. Cell preparations from the shocked and unshocked cultures were assayed for GS activity at a variety of pH values.

3.2.3. ASSAYS FOR GS ACTIVITIES

Bacterial GSs catalyze a variety of reactions (Kleinschmidt and Kleiner, 1978).

$$\text{Glutamate} + NH_3 + \text{ATP} \longrightarrow \text{Glutamine} + \text{ADP} + P_i$$

(Biosynthetic reaction)

$$\text{Glutamate} + NH_2OH + \text{ATP} \longrightarrow \gamma\text{-Glutamylhydroxamate} + \text{ADP} + P_i$$

(Synthetic or forward transferase reaction)

Glutamine + NH_2OH ⟶ γ-Glutamylhydroxamate + NH_3

(Transferase reaction)

All reactions are dependent on the presence of Mg^{2+} or Mn^{2+} in the reaction medium. The transferase reaction requires in addition arsenate and adenosine-5'-diphosphate (ADP) as cofactors. The GS assays for the respective enzyme forms are summarized in Table 3.2.1.

The transferase reaction has several advantages over the biosynthetic and synthetic reactions: it is catalytically less complex, having no requirement for ATP activation (Shapiro and Stadtman, 1970). The transferase reaction is useful during purification of GS, or in studying activities in cell extracts, since it is less subject to interference by contaminating enzymes than is the biosynthetic reaction and it is apparently unaffected by interaction between adenylylated and unadenylylated subunits. The purified enzyme is usually assayed by measuring the production of inorganic phosphate in the biosynthetic reaction, and should be applied only to extracts free from ATP-ase. The γ-glutamyl transferase activity of GS has as yet no known physiological function (Wohlhueter et al., 1973).

BIOSYNTHETIC ASSAY

The biosynthetic activity of GS was assayed according to the method of Shapiro and Stadtman (1970). The enzyme is assayed by measuring the production of inorganic phosphate (P_i) in the biosynthetic reaction (Section 3.2.3).

TABLE 3.2.1: ASSAYS FOR GS ACTIVITIES

ASSAY	MEASUREMENT OF GS
GS_T	Total enzyme
GS_B: Mg^{2+}-dependent	Unadenylylated enzyme
Mn^{2+}-dependent	Adenylylated enzyme
Forward Transferase	Unadenylylated enzyme
Inhibition of GS_T by 60 mM Mg^{2+}	Unadenylylated enzyme

Samples (0.1 ml) of enzyme were added to 0.1 ml of a reaction mixture containing 0.1 ml of 1.0 M imidazole-HCl buffer (pH 7.0), 0.25 ml of 0.06 M sodium ATP (pH 7.0), 0.2 ml of 1.0 M sodium glutamate (pH 7.0), 0.1 ml of 1.0 M NH_4Cl; and 0.06 ml of 1.67 M $MgCl_2$ (or 0.1 ml of 0.1 M $MnCl_2$ when this activator is used). The two-fold concentrated assay mixture was prepared to a final volume of 1.0 ml. The enzyme reaction was allowed to proceed for 15 min at 37°C. The amount of enzyme was adjusted so that not more than 0.25 µmol of phosphate was produced in the reaction, which was terminated by the addition of 1.8 ml of fresh ferrous sulphate reagent ($FeSO_4 \cdot 7H_2O$ was dissolved in 0.015 N H_2SO_4 to a final concentration of 0.8%). This was followed by the addition of 0.15 ml of ammonium molybdate reagent (6.6% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 7.5 N H_2SO_4), the tubes were vigorously mixed, and the colour allowed to develop for 1 min. The resulting colour was read at 660 nm on a MSE-spectrophotometer. Corrections were made for non glutamate-dependent ATP hydrolysis by assaying appropriate mixtures during the assay procedure. One unit of GS biosynthetic activity is defined as the amount of enzyme that results in an increase in absorbance at 660 nm of 0.1 in 15 min at 37°C.

γ-GLUTAMYL TRANSFERASE (γGT) ASSAY

The γGT assay measures the total amount of GS present, since both the adenylylated and deadenylylated forms of GS are active in this assay (Stadtman et al., 1970). GS transferase activity was assayed according to the method of Shapiro and

Stadtman (1970) as modified by Bender et al. (1977). A fresh concentrated assay mixture was prepared daily by mixing the stock solution in the order and proportions shown in Table 3.2.2. This procedure avoided the formation of precipitates and resulted in higher reproducibility of the assay. A blank lacking ADP and arsenate was included in the assay. CTAB, which rendered the cell permeable to the reactants, was replaced with water when crude extracts rather than whole cells were being assayed. The pH of the reaction mixture was adjusted with either 1 M HCl or 2 M KOH. After the pH had been adjusted, the reaction mixture was placed at 4°C if it was not used immediately. Samples (0.1 ml) were added to 0.4 ml of the concentrated assay mixture. The solution was equilibrated for 5 min at 37°C, and the reaction was initiated by the addition of 0.05 ml of 0.20 M L-glutamine to each tube. After 15 min at 37°C, the reaction was terminated by the addition of 1.0 ml of "stop mix" containing 55 g of $FeCl_3 \cdot 6H_2O$, 20 g of TCA, and 21 ml of concentrated HCl per litre. The resulting colour denoting the production of γ-glutamylhydroxamate was read at 540 nm on an MSE-spectrophotometer.

FORWARD TRANSFERASE ASSAY

The forward reaction assay measures the ability of GS to form glutamine. The concentrated assay mixture was prepared daily by mixing the stock solution in the order and proportions shown in Table 3.2.2. It was adjusted to the required pH with 10 M KOH. Samples (0.1 ml) were added to 0.4 ml of the

TABLE 3.2.2. COMPOSITION OF GS REACTION MIXTURES (BENDER ET AL., 1977)

Preparation	Volume (ml)	Stock solution
GS_T assay mixture	7.53	Water
	2.25	1.0 M imidazole-HCl, pH 7.15
	0.37	0.80 M hydroxylamine-HCl
	0.045	0.10 M $MnCl_2$
	1.5	0.28 M potassium arsenate, pH 7.15
	0.15	40 mM sodium ADP, pH 7.0
	1.5	CTAB (1 mg/ml)
Forward-transferase assay mixture	7.2	Water
	2.0	1.0 M imidazole-HCl, pH 7.15
	1.25	0.80 M hydroxylamine-HCl
	0.40	3.0 M $MgCl_2$
	4.2	0.85 M monosodium L-glutamate
	2.0	CTAB (1 mg/ml)

reaction mixture. After 5 min of equilibration at 37°C, the reaction was initiated by the addition of 0.06 ml of 0.2 M ATP, adjusted to pH 7.7 with KOH. After 15 min incubation at 37°C, the reaction was terminated by the addition of 1 ml of stop mix. The samples were centrifuged to remove any precipitate, and the absorbance at 540 nm was measured as in the GS_T assay. Blanks without ATP were included in the assay.

TRANSFERASE INHIBITION ASSAY

A simple method for estimating the average state of adenylylation of GS involved measuring the GS_T activity under two conditions (Stadtman and Ginsburg, 1974):

a) in the presence of 0.3 mM Mn^{2+}, the GS_T assay

b) in the presence of 0.3 mM Mn^{2+} + 60 mM Mg^{2+}

The second assay was a measure of the unadenylylated enzyme only. Therefore the average state of adenylylation ($\bar{n}$) was calculated from the ratio of the two measurements as follows:

$$E_{\bar{n}} = 12 - 12\frac{b}{a} .$$

One unit of GS activity obtained using the GS_T, forward transferase and GS_T + 60 mM Mg^{2+} assays is defined as the amount of enzyme that results in an increase in absorbance of 0.1 at 540 nm in 15 min at 37°C.

3.2.4. ASSAYS FOR GDH AND GOGAT ACTIVITIES

GDH and GOGAT activities were assayed according to the method of Meers et al. (1970), but with modifications. Activities

were determined spectrophotometrically by measuring the oxidation of NADPH at a wavelength of 340 nm. The reaction mixture contained 10 mM α-KG, 0.6 mM NADPH and either 80 mM NH_4Cl or 10 mM L-glutamine for assaying GDH and GOGAT respectively in a final volume of 3.0 ml of 100 mM Tris-HCl buffer (pH 7.6). Sonic extracts (0.15 ml) were added to 0.15 ml of the reaction mixture, maintained at 22°C to initiate the reaction which was allowed to proceed for 8 min at 22°C. The absorbance readings at 340 nm were recorded on a DU-8 spectrophotometer. One unit of GDH or GOGAT activity is defined as the amount of enzyme that results in a decrease in absorbance of 0.1 at 340 nm in 8 min at 22°C.

3.3. RESULTS

3.3.1. BUFFER PREFERENCE

The effect of 0.05 M K_2PO_4 and 10 mM imidazole + 2.5 mM $MgCl_2$ buffers on GS_T activity was investigated (Table 3.3.1). Crude extracts prepared from cultures containing L-alanine and L-arginine as sole nitrogen sources were tested for GS_T activity. There was a definite buffer preference and extracts in 10 mM imidazole + 2.5 mM $MgCl_2$ buffer exhibited an almost 10-fold increase in GS activity than the same extracts in 0.05 M K_2PO_4 buffer. The imidazole buffer (pH 7.15) was used in all further experiments.

TABLE 3.3.1. EFFECT OF PHOSPHATE AND IMIDAZOLE BUFFERS ON GS-TRANSFERASE ACTIVITY IN V. ALGINOLYTICUS

MEDIUM	CONCENTRATION (mM)	GS_T ACTIVITY (units ml^{-1})	
		Phosphate	Imidazole
Ala-SMM	20	0.28	2.19
Arg-SMM	20	0.14	1.39

3.3.2. EFFECT OF DIVALENT CATION SPECIFICITY AND CONCENTRATION ON GS_B ACTIVITY

Donohue and Bernlohr (1981) reported that Mg^{2+} and Mn^{2+} were the preferred cations for GS activity in many micro-organisms. The effect of various concentrations of Mg^{2+} and Mn^{2+} ions on GS_B activity was studied (Table 3.3.2). Crude extracts from a stationary phase culture grown in proline-SMM (43 mM) was used. A culture in transition from exponential to stationary phase is a natural example of adenylylation *in vivo* (Wohlhueter *et al.*, 1973).

GS_B activity decreased with increasing concentrations of Mg^{2+} and Mn^{2+} ions. It was found that with Mn^{2+} ion concentrations higher than 0.5 M, a precipitate resulted. The Mn^{2+}-dependent GS_B activity was slightly higher than the Mg^{2+}-dependent activity. The optimum Mg^{2+} and Mn^{2+} ion concentrations of 1.67 M and 0.1 M respectively were used in all experiments.

3.3.3. NH_4^+-SHOCKING AND ITS EFFECT ON GS ACTIVITY

Crude extracts of unshocked and shocked (15 mM NH_4^+) cells were tested using the GS_T, GS_B, forward transferase and GS_T + 60 mM Mg^{2+} assays.

NH_4^+-shocking of *V. alginolyticus* cells grown in glu-SMM resulted in a reduction in GS_T activity. To establish that this effect was not due to a shift in the pH-optimum of the

TABLE 3.3.2. EFFECT OF DIVALENT CATION SPECIFICITY AND CONCENTRATION ON GS_B ACTIVITY IN V. ALGINOLYTICUS

CATION	CONCENTRATION (M)	GS_B ACTIVITY (units ml^{-1})
Mg^{2+} :	1.67	6.0
	5.0	5.6
	10.0	4.5
Mn^{2+} :	0.1	7.5
	0.2	7.1
	0.3	6.2
	0.5	5.8

enzyme, pH-GS_T activity profiles of crude extracts from shocked and unshocked cultures were obtained (Fig. 3.3.1). The pH optimum of shocked and unshocked extracts was 7.9. At this pH value, GS_T activity of the extract from unshocked cells was 1.9-fold higher than the shocked sample. The optimum pH value for GS_T activity was used in all experiments. The GS_T activity of the extract from shocked cells was lower than the extract from unshocked cells at all the pH values tested. In the pH range tested, no isoactivity point could be defined.

pH profiles obtained for unshocked and shocked whole cell preparations treated with CTAB were similar to those obtained using crude extracts.

Dialysis of crude extracts from unshocked and shocked cells against imidazole-$MgCl_2$ buffer for 18 h did not affect the GS_T activities obtained at the optimum pH.

The inhibition of GS_T activity by 60 mM Mg^{2+} could not be demonstrated for V. alginolyticus. The low pH of the assay mixture could not be adjusted due to the formation of a heavy precipitate. The 60 mM Mg^{2+} inhibition of GS_T activity has been shown to correlate with a high degree of adenylylation in E. coli (Stadtman et al., 1970).

Since the inhibition of GS_T activity by Mg^{2+} could not be used for V. alginolyticus GS, an attempt was made to measure the amount of unadenylylated enzyme in crude extracts by using

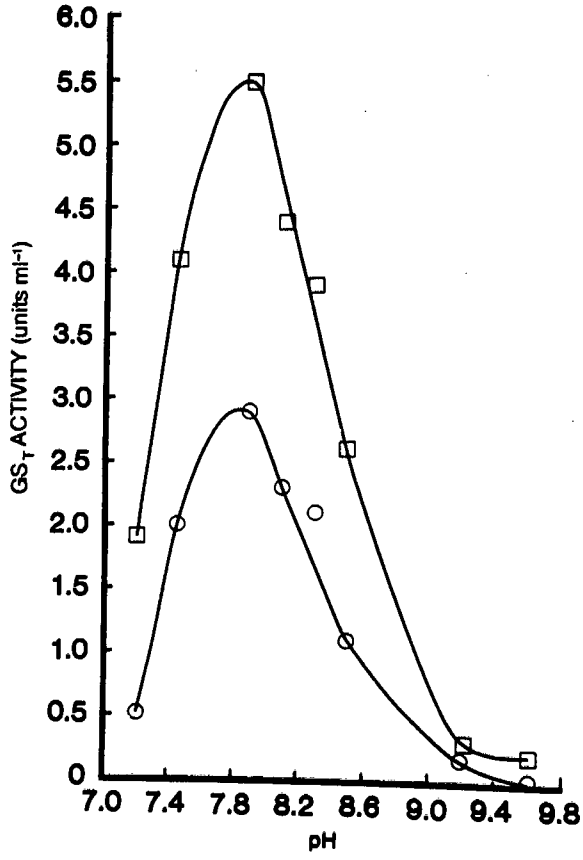


FIG. 3.3.1. GS_T Activity profiles of crude extracts prepared from unshocked (□) and shocked (+15 mM NH_4^+) (○) exponential phase cultures of V. alginolyticus, grown in glu-SMM (34 mM).

the forward transferase assay. This reaction was attempted at various pH values, but absorbance readings at 540 nm were extremely low. The forward transferase reaction for GS could also not be demonstrated using purified GS (P. Brandt, personal communication). The absorbance at 540 nm using the GS_T assay was 0.61 at pH 7.9. Absorbance readings for the same GS purified preparation using the forward transferase assay was 0.0 and 0.08 at pH 7.6 and pH 8.15 respectively. A range of pH values were then used for the forward transferase assay but absorbance readings at 540 nm were still low.

Crude extracts prepared from shocked and unshocked cultures when assayed for GS_B activity showed higher Mg^{2+}- than Mn^{2+}-dependent activity in both extracts (Table 3.3.3). Both the Mg^{2+}- and Mn^{2+}-dependent activities were higher in the unshocked than the shocked sample.

NH_4^+-shocking of V. alginolyticus cultures resulted in a decrease in GS_T activity (Fig. 3.3.1) and both the Mg^{2+} and Mn^{2+}-dependent GS_B activities (Table 3.3.3).

3.3.4. EFFECT OF AMMONIUM IONS AND GLUTAMATE ON GS_T ACTIVITY

Crude extracts prepared from cultures containing varying concentrations of ammonium ions were tested for GS_T activity (Table 3.3.4). The enzyme was repressed by growth on media containing readily available nitrogen, that is high concentrations of ammonium salts. An inverse relationship existed

TABLE 3.3.3. Mg^{2+}- AND Mn^{2+}-DEPENDENT GS_B ACTIVITIES OF CRUDE EXTRACT PREPARED FROM UNSHOCKED AND SHOCKED CULTURES OF V. ALGINOLYTICUS GROWN IN GLU-SMM (34 mM)

CRUDE EXTRACT	GS ACTIVITY (units ml^{-1})	
	Mg^{2+}	Mn^{2+}
Unshocked sample	6.3	3.8
Shocked sample	2.5	1.9

TABLE 3.3.4. EFFECT OF AMMONIUM IONS AND GLUTAMATE ON GS_T LEVELS

MEDIUM	CONCENTRATION OF NITROGEN SOURCE (mM)	GS_T ACTIVITY (units ml^{-1})
ASMM	20	1.4
ASMM	5	2.3
ASMM	2.5	6.4
Glu-SMM	20	7.0
ASMM + Glu	20 20	1.2

between ammonium availability and GS activity. GS activity increased when the ammonium concentration was decreased to a level where it became the limiting factor for growth. High levels of GS activity were present in glutamate-grown cells and a low level of enzyme was found in cells grown on a combination-medium of glutamate and high ammonium salts.

3.3.5. EFFECT OF AMINO ACIDS ON GS_T ACTIVITY

Various amino acids were used as sole nitrogen sources for growth of V. alginolyticus (Table 3.3.5). Crude extracts prepared from these cultures were assayed for GS_T activity. V. alginolyticus grew at different rates in the presence of different nitrogen sources. The relative differences in growth rate promoted by the various nitrogen sources were reproducible. However, all assays were performed at standardized cell densities. The highest levels of GS_T activity were obtained when proline was used as the sole nitrogen source. This was probably due to the poor availability of proline-nitrogen for growth. The use of amino acids proline, leucine, isoleucine, tryptophan, histidine, glutamic acid, alanine, glycine and arginine as sole nitrogen sources resulted in increased levels of GS_T activity. An interesting result was obtained when glutamine was used as a source of nitrogen. There was a marked decrease in the level of GS_T activity. Glutamine repressed the level of GS_T activity more severely than $(NH_4)_2SO_4$, at an almost equal concentration.

TABLE 3.3.5. EFFECT OF AMINO ACIDS ON GS_T ACTIVITY IN V. ALGINOLYTICUS

Amino acids were added at 0.5% (w/v).

Nitrogen Source	Concentration (mM)	GS_T Activity (units ml^{-1})
$(NH_4)_2SO_4$	38	1.3
L-proline	43	46.7
L-leucine	38	16.9
L-isoleucine	38	16.4
L-tryptophan	25	13.7
L-histidine	24	11.1
L-glutamic acid	34	7.0
L-alanine	56	7.7
L-glycine	67	5.4
L-arginine	24	4.6
L-glutamine	34	0.2

3.3.6. EFFECT OF AMINO ACIDS AND AMMONIUM IONS ON GS_B ACTIVITY

Crude extracts prepared from cultures grown in various amino acids were tested for Mg^{2+}- and Mn^{2+}-dependent activities (Table 3.3.6). The results resembled those obtained using the GS_T assay (Table 3.3.5). There was little or no distinction between GS_B levels obtained using either the Mg^{2+} or Mn^{2+}-dependent assay.

3.3.7. EFFECT OF GLUCOSE AND CYCLIC AMP ON GS_T LEVELS

The effect of glucose and cyclic AMP on GS_T levels was investigated (Table 3.3.7). The result was surprising. The addition of glucose resulted in a marked increase in GS_T activity in cells grown in glutamate (34 mM) and in cells grown in NH_4^+ (7.7 mM) as sole nitrogen sources. The stimulatory effect was almost 4-fold with the former extract and 2-fold with the latter sample. Cyclic AMP had little effect on this glucose-dependent increase in GS_T activity in cells grown in glu-SMM.

3.3.8. EFFECT OF CARBON SOURCE ON GS_T ACTIVITY

The unusual result with glucose prompted an examination of the effects of other carbon sources on GS_T activity (Table 3.3.8). The addition of sucrose, fructose, glycerol, glucose and maltose all stimulated GS_T activity. Arabinose and lactose repressed GS_T activity.

TABLE 3.3.6. EFFECT OF AMINO ACIDS AND AMMONIUM IONS ON GS_B ACTIVITY IN CRUDE EXTRACTS PREPARED FROM V. ALGINOLYTICUS CELLS.

NITROGEN SOURCE	CONCENTRATION (mM)	GS_B ACTIVITY (units ml^{-1})	
		Mg^{2+}	Mn^{2+}
$(NH_4)_2SO_4$	38	0.8	0.6
L-histidine	24	8.0	69
L-glutamic acid	34	8.5	8.2
L-proline	43	12.2	11.0

TABLE 3.3.7. EFFECT OF GLUCOSE AND CYCLIC AMP ON GS_T LEVELS IN MID-EXPONENTIAL PHASE CELLS OF V. ALGINOLYTICUS GROWN IN GLU-SMM (34 mM) AND ASMM (7.7 mM)

MEDIUM	GLUCOSE (mM)	CYCLIC AMP (mM)	GS_T ACTIVITY (units ml^{-1})
Glu*-SMM	0	0	7.5
Glu-SMM	11	0	28
Glu-SMM	11	5	29
ASMM	0	0	4.7
ASMM	11	0	9.5

*Glu represents 34 mM glutamate.

TABLE 3.3.8. EFFECT OF CARBON SOURCE ON GS_T LEVELS OF V. ALGINOLYTICUS CELLS GROWN IN GLU-SMM (34 mM GLUTAMATE).

Carbon sources other than succinate were added at 0.5% (w/v).

CARBON SOURCE	CONCENTRATION (mM)	GS_T ACTIVITY (units ml^{-1})
Succinate	9	4.0
Sucrose	15	46.3
Fructose	27	40.1
Glycerol	54	28.4
Glucose	27	28.0
Maltose	14	13.4
Arabinose	33	3.0
Lactose	14	1.0

3.3.9. SENSITIVITY OF GS_T ACTIVITY TO FEEDBACK MODIFIERS

To determine whether feedback inhibition was also a property of GS from *V. alginolyticus*, the effect of amino acids on GS_T activity in crude extract prepared from cells grown in 2.5 mM $(NH_4)_2SO_4$ - SMM was investigated (Table 3.3.9). Compounds tested as feedback modifiers were added to the reaction mixes prior to the addition of the crude extract, and controls for each effector were included in the assay.

The enzyme was subjected to feedback inhibition by glycine, AMP, tryptophan, arginine, and $(NH_4)_2SO_4$. Inhibition by these compounds was in the order of 37 - 47%. Glutamic acid, leucine, isoleucine, histidine and alanine had little effect on GS_T activity. The maximum inhibition caused by these effectors was 21%. Glutamine stimulated GS_T activity of cell-free extracts of *V. alginolyticus*.

3.3.10. SENSITIVITY OF GS_T ACTIVITY TO MULTIPLE FEEDBACK MODIFIERS

To determine the pattern of feedback inhibition in *V. alginolyticus*, the inhibition caused by multiple inhibitors was examined (Table 3.3.10). The addition of both arginine and glycine together resulted in 58% inhibition of GS_T activity while the simultaneous addition of glycine, arginine and AMP resulted in an inhibition of 64% (Table 3.3.10). Arginine glycine, AMP and tryptophan together caused 84% inhibition; the addition of glutamine to these compounds reduced this to 61% inhibition.

TABLE 3.3.9. SENSITIVITY RESPONSE OF GS_T ACTIVITY TO FEEDBACK MODIFIERS.

Crude extract was prepared from V. alginolyticus cells grown on 2.5 mM $(NH_4)_2SO_4$ as the sole source of nitrogen. GS_T activities were expressed as a percentage of the activity in 2.5 mM $(NH_4)_2SO_4$-SMM.

EFFECTOR	CONCENTRATION (mM)	GS_T ACTIVITY (%)
None		100
L-glutamine	34	203
L-proline	43	109
L-glutamic acid	34	95
L-leucine	38	89
L-isoleucine	38	84
L-histidine	24	79
L-alanine	56	79
L-glycine	67	63
AMP	3.5	59
L-tryptophan	25	59
L-arginine	24	54
$(NH_4)_2SO_4$	38	53

TABLE 3.3.10. FEEDBACK INHIBITION ON CELL FREE EXTRACT OF V. ALGINOLYTICUS GS BY MULTIPLE INHIBITORS.

Inhibitors were added at final concentrations shown in Table 3.3.9. Cumulative inhibition was calculated by the method of Woolfolk and Stadtman (1967)

FEEDBACK MODIFIER/S	% INHIBITION		
	OBSERVED	ADDITIVE	CUMULATIVE
L-arginine (A)	46	-	-
L-glycine (B)	37	-	-
AMP (C)	41	-	-
L-tryptophan (D)	41	-	-
L-glutamine (E)	0	-	-
A + B	58	83	66
A + B + C	64	124	80
A + B + C + D	84	165	88
A + B + C + D + E	61	-	-

3.3.11. EFFECT OF NITROGEN SOURCE AND GLUCOSE ADDITION ON THE PRODUCTION OF GDH AND GOGAT

The production of GOGAT was low in a medium containing excess nitrogen (50 mM NH_4^+), while GDH activity was markedly derepressed (Table 3.3.11). The opposite situation existed in a medium containing a growth rate-limiting source of nitrogen (2.5 mM NH_4^+). Synthesis of GDH was repressed and GOGAT production was increased. The addition of glucose to the nitrogen-limited medium did not affect the low level of GDH but repressed the high level of GOGAT production. No conclusion can be drawn as to the effect of glucose on GDH as it was already repressed. In a medium containing a high concentration of glutamate, the production of both GDH and GOGAT was low.

GDH was found to be NADP-specific as no reaction was observed when NAD was used in extracts prepared from a medium containing 50 mM $(NH_4)_2SO_4$ as the nitrogen source, conditions which would usually result in derepression of GDH production.

TABLE 3.3.11. EFFECT OF NITROGEN SOURCE AND GLUCOSE (11 mM) ADDITION ON THE PRODUCTION OF GDH AND GOGAT IN V. ALGINOLYTICUS

MEDIUM	CONCENTRATION OF NITROGEN SOURCE (mM)	ENZYME LEVELS (units ml^{-1})	
		GDH	GOGAT
$(NH_4)_2SO_4$ - SMM	50	13	4.6
$(NH_4)_2SO_4$ - SMM	2.5	3.5	10.6
$(NH_4)_2SO_4$ - SMM + GLUCOSE	2.5	3.6	2.9
Glu - SMM	50	4.7	5.5

3.4. DISCUSSION

3.4.1. GENERAL

Studies by Hubbard and Stadtman (1967) have shown that differences occur in the regulation of GSs in various microorganisms. Results obtained with crude extracts of *V. alginolyticus* containing GS indicated that the regulatory mechanisms governing GS might be different from those of *E. coli*. The detailed study of enzymes from different organisms might offer new insights into biological control mechanisms.

The *in vivo* flux through a given metabolic reaction can be regulated by enzyme quantity; by the relative concentrations of substrates, products, activators, and inhibitors; and by the availability of cofactors, metal ions, and any other factors which influence enzyme activity (Donohue and Bernhohr, 1981). The data compiled here support the conclusion that many of these mechanisms are operative *in vivo* to regulate glutamine synthesis at the level of GS during growth of *V. alginolyticus*.

3.4.2. EFFECT OF BUFFER AND Mg^{2+} AND Mn^{2+} CATIONS ON GS ACTIVITY

Crude extracts of GS from *V. alginolyticus* resuspended in imidazole-$MgCl_2$ buffer resulted in higher levels of GS_T activity than cells resuspended in phosphate buffer.

The concentration of divalent cation present in the assay

system affected both Mg^{2+}- and Mn^{2+}-dependent GS_B activities but only to a slight extent. The Mn^{2+}-dependent GS_B activities were slightly higher than the Mg^{2+}-supported activities. This result is consistent with the existence of an adenylylation system as the Mn^{2+} ion favours the adenylylated enzyme and cells are usually adenylylated at stationary phase as ammonium accumulates at the end of the logarithmic growth phase. However, from data obtained using crude extracts of exponential phase cells of V. alginolyticus it seemed that Mg^{2+} was the preferred cation for both shocked and unshocked cultures. A Mn^{2+}-dependent increase in activity should have occurred on the addition of NH_4^+. Furthermore, growth of V. alginolyticus cells in various nitrogen sources resulted in similar Mg^{2+}- and Mn^{2+}-supported GS_B activities for a particular source of nitrogen. In this respect, V. alginolyticus seemed to resemble B. subtilis and not the enteric bacteria. In B. subtilis either Mg^{2+} or Mn^{2+} can activate GS_B activity (Deuel and Stadtman, 1970). The ability of the GS protein to be activated by Mg^{2+} and by Mn^{2+} is an intrinsic property of the enzyme independent of growth conditions and is not determined by adenylylation of the enzyme as it is for E. coli (Deuel and Stadtman, 1970). From these data, it can be concluded that the GS_B reaction cannot satisfactorily be utilized when assaying crude extract.

Purified preparations of GS from V. alginolyticus responded differently to Mg^{2+} and Mn^{2+} cations (P. Brandt, personal communication). Presumably adenylylated enzyme treated with snake venom phosphodiesterase

(SVP) resulted in high Mg^{2+}-dependent activity consistent with the removal of AMP residues. The Mn^{2+}-dependent activity of treated samples was low. Untreated enzyme (-SVP) had higher Mn^{2+}- than Mg^{2+}-supported activity. Thus, an adenylylation-deadenylylation is operative in V. alginolyticus but could not be detected by Mg^{2+} and Mn^{2+}-dependent Gs_B assays on ammonium-shocked and unshocked crude extracts.

3.4.3. EFFECT OF NH_4^+-SHOCKING ON GS_T ACTIVITY

On addition of $(NH_4)_2SO_4$ to V. alginolyticus cells growing in glu-SMM, an NH_4^+-induced loss of Mn^{2+}-dependent GS_T activity was observed. This was unusual as the GS_T activity should not be affected (Gancedo and Holzer, 1968). It was established that the apparent inactivation of GS was not due to a shift in the pH optimum. The pH-GS_T activity profiles of crude extracts prepared from unshocked and shocked cultures of V. alginolyticus followed a similar pattern but the GS_T activities of shocked cells were lower at all pH values examined. The pH optimum was the same for the shocked and unshocked samples. In E. coli the pH optimum for catalysis of γ-glutamyl transfer differs markedly in the two enzyme forms, being 6.9 for the adenylylated preparation and 7.9 for the unadenylylated form (Stadtman and Ginsburg, 1974). pH-GS_T activity profiles for the phototrophs Rhodopseudomonas palustris (Alef and Zumft, 1981) and Chloroflexus aurantiacus (Kaulen and Klemme, 1983) resembled those for V. alginolyticus and an isoactivity point could also not be defined from these curves.

Falk et al. (1982) and Yoch et al. (1983) also reported an NH_4^+-dependent decrease in GS_T activity in "shocking" experiments on Rhodospirillum rubrum. They suggest that the GS of R. rubrum is regulated in response to NH_4^+ by both an adenylylation system (Davies and Ormerod, 1982) and an undefined system not related to adenylylation. This raises the question as to whether a similar situation could exist in V. alginolyticus. The mechanism responsible for this NH_4^+-dependent decrease in GS_T activity is not known. The GS_T activity of presumably adenylylated enzyme could not be specifically inhibited in the presence of 60 mM Mg^{2+}; both shocked and unshocked samples were severely inhibited due to the low pH at which the assay was performed. At higher pH values, Mg^{2+}-arsenate precipitates resulted.

At present there seems to be no explanation for the rapid reduction in Mn^{2+}-dependent GS_T activity. It was not due to a change in the pH optimum of the enzyme. The reduction in activity may have been due to feedback inhibition by $(NH_4)_2SO_4$. When $(NH_4)_2SO_4$ was added to cell-free extracts of V. alginolyticus, prepared from cultures grown in a derepressing medium, GS_T activity was feedback inhibited. This effect should perhaps be distinguished from the long term regulation at the genetic level of repression and derepression of GS prepared from cells grown in the presence of excess $(NH_4)_2SO_4$.

The synthesis of activating or inactivating enzymes for

regulatory purposes appears to be more expensive for the cell than regulation by repression, induction or feedback inhibition (Gancedo and Holzer, 1968). The GS activity of Sarcina citrea, B. subtilis, Saccharomyces cerevisiae, Candida utilis and Lactobacillus plantarum are not affected by NH_4^+-shocking (Gancedo and Holzer, 1968). They suggest that it might be possible that adenylylation of GS is a relic of a primitive system which in most other organisms has been lost and replaced by other control mechanisms. Baumann and Baumann (1980) reported that the GS of the marine bacterium Beneckea alginolytica strain 90 [Vibrio consists mainly of species previously in the genus Beneckea (Baumann et al., 1980)] is regulated by adenylylation and deadenylylation; but they were also unable to find an iso-activity point using the GS_T assay. Treatment with SVP released the enzyme from inhibition by Mg^{2+} and resulted in stimulation of activity by this cation. The results of SVP treatment are therefore consistent with a mechanism of regulation of activity by adenylylation. Baumann and Baumann (1980) have purified the GS of V. alginolyticus 90 with a 10% recovery but give no details of its characterization. Immunological comparisons of the GSs from V. alginolyticus 90 and E. coli indicate that species of Vibrio have diverged considerably from E. coli and they suggest four major groupings among these genera: Vibrio, Photobacterium, Aeromonas and a large heterogenous group which included the peritrichously flagellated terrestrial enterobacteria (Baumann et al., 1980). It would be interesting to look for immunological cross-reactions between enzymes, which

suggest antigenic homology, using GS antisera of both Gram-positive and Gram-negative bacteria against the GS of V. alginolyticus.

3.4.4. FEEDBACK INHIBITION

The GS_T activity of V. alginolyticus GS was subject to feedback inhibition by glycine, AMP, tryptophan, arginine, and $(NH_4)_2SO_4$ and to a lesser extent by glutamic acid, leucine, isoleucine, histidine and alanine. Deuel and Prusiner (1974) found that the GS of B. subtilis was inhibited by AMP, tryptophan, glycine, histidine, alanine and cytidine triphosphate (CTP). Studies by Woolfolk and Stadtman (1967) on the GS of E. coli showed that feedback inhibition was effected by alanine, glycine, histidine, tryptophan, AMP, carbamyl phosphate, glucosamine-6-phosphate and CTP. However, the sensitivity of GS to feedback inhibition was found to be altered by adenylylation (Ginsburg and Stadtman, 1973). Feedback inhibition by $(NH_4)_2SO_4$ which occurred in V. alginolyticus has also been reported for Thiobacillus neapolitanus (Beudeker et al., 1982). The stimulatory effect of glutamine on cell-free extracts of V. alginolyticus was probably due to its action as a substrate for the transferase reaction (Section 3.2.3) which overcomes its ability to feedback inhibit. The range of compounds acting as feedback modifiers of GS seems fairly similar for the various species. Histidine, AMP, tryptophan, CTP, glucosamine-6-phosphate and carbamyl phosphate are compounds whose biosyntheses utilize the amide group of glutamine, and alanine and glycine arise from glutamine-

dependent transamination reactions (Shapiro and Stadtman, 1970). Inhibition of GS of E. coli was rather specific and 60 other metabolites tested were without effect, except for closely related analogues of the eight inhibitors listed above (Shapiro and Stadtman, 1970).

The effect of more than one inhibitor on GS_T activity of V. alginolyticus cell-free extract prepared from cells grown in a derepressing medium was also investigated. The total inhibition caused by a mixture of inhibitors was less than the sum of the inhibitory effects caused by each of the inhibitors tested individually. Woolfolk and Stadtman (1967) suggest that less than additive inhibition would result if there is antagonism between the various inhibitors, or if each of the inhibitors is completely independent in its action on the enzyme. From the data, it can be seen that multiple inhibitions are not additive. The observed inhibition is less than the calculated cumulative inhibition, indicating that these modifiers interact antagonistically (Wedler et al., 1976). Antagonistic effects may be interpreted as being due to either overlapping modifier domains or separate but antagonistically interacting sites (Wedler et al., 1976).

Stadtman and Ginsburg (1974) found that relatively high concentrations (1-100 mM) of most of the inhibitors of the E. coli GS are required to produce 50% inhibition of enzymic activity. They state that therefore under even extreme

physiological conditions, each inhibitor will cause only partial inhibition of GS activity. Hubbard and Stadtman (1967) conclude that from the remarkable similarity seen with GS from many microorganisms, feedback inhibition of GS by multiple end-products of glutamine metabolism is a general regulatory phenomenon that has been conserved through evolution. However, they also state that it has been known for closely related species to exhibit marked differences in the patterns of feedback of an enzyme which presumably occupies the same relative position in their metabolism.

3.4.5. EFFECT OF NITROGEN AND CARBON SOURCE ON GS_T ACTIVITY IN V. ALGINOLYTICUS

The activity of GS varied greatly in V. alginolyticus depending upon the nitrogen supply utilized and also the carbon source in the growth medium. Results indicate that the formation of GS is subject to repression by high concentrations of ammonium salts but is derepressed if the level of ammonium salts is growth rate-limiting. Cells grown in proline, leucine, isoleucine, tryptophan, histidine, glutamic acid, glycine and arginine had high levels of GS activity. This implies that growth of V. alginolyticus under these different conditions resulted in some type of nitrogen limitation which in turn regulated GS synthesis (Donohue and Bernlohr, 1981). The highest level of GS activity was obtained when proline was the sole nitrogen source and is probably due to derepression of the enzyme as a result of the poor availability

of proline nitrogen for growth. Enhancement of GS activity in extracts prepared from cells grown in various amino acids has been reported for Chloroflexus aurantiacus (Kaulen and Klemme, 1983) and Azotobacter vinelandii (Lepo et al., 1982). Donohue and Bernlohr (1981), from studies on B. licheniformis A5 GS state that the actual nature of the signal of nitrogen limitation is unknown but indicate that the signal of nitrogen limitation is not reflected in the intracellular pool of ammonia or glutamine. Results obtained by Schreier et al. (1982) in their study on the nitrogen regulatory enzymes in Bacillus spp. indicate that basal levels of the enzymes were regulated by the nitrogen source present and the mechanism of this control is unknown. Furthermore, a comparison of the intracellular pool sizes of ammonia, glutamate, and α-KG with the ammonia assimilatory enzyme levels showed that the pools of these metabolites did not change in a manner consistent with their use as regulators of the synthesis of any of these enzymes (Schreier et al., 1982).

In contrast to E. coli, the GSs of Bacillus (Hubbard and Stadtman, 1967; 1967a), Clostridium (Hubbard and Stadtman, 1967) and Lactobacillus (Ravel et al., 1965), all Gram-positive organisms, are inhibited by glutamine. The GS of V. alginolyticus was severely repressed when L-glutamine was the sole nitrogen source in the medium. However, the transferase activity of the GS of V. alginolyticus in cell-free extracts was actually stimulated by glutamine indicating that the repressive effect when glutamine is present in the growth medium is at the level of production of the enzyme.

In *B. subtilis* (Pan and Coote, 1979) and in *V. alginolyticus* growth of cells on glutamine inhibited GS_T activity to a greater extent than growth on $(NH_4)_2SO_4$ as sole nitrogen source, at almost equal concentrations.

The ability of NH_4^+ to repress GS activity is reasonable since NH_4^+ at high concentrations can replace the amide group of glutamine as the nitrogen donor in most glutamine-dependent biosynthetic reactions (Stadtman and Ginsburg, 1974). Therefore when NH_4^+ is available at very high concentrations, the requirement for glutamine is greatly reduced, probably to a level needed only for its unique function as a building block in protein synthesis (Stadtman and Ginsburg, 1974). Since glutamine is used for a variety of metabolic functions, it is not surprising that GS would be regulated by a mechanism which responds specifically to cellular needs for glutamine. Therefore, from the standpoint of cellular regulation, it seems that for *E. coli* and *B. subtilis*, the intracellular level of glutamine is the most important factor in determining GS activity (Deuel and Stadtman, 1970). In *B. subtilis* glutamine directly represses GS activity, whereas in *E. coli* glutamine exerts its effect indirectly, through the activation and inhibition of adenylylated and deadenylylated enzymes, respectively. The two types of regulation achieve somewhat similar results although this product inhibition by L-glutamine seems a greatly simplified mechanism to regulate overall glutamine synthesis when compared to the complex cascade enzyme system employed by *E. coli*. In mammals direct inhibition of GS by L-glutamine is also observed (Deuel *et al.*, 1973); when glutamine

is added to the cell culture medium, a marked decrease in GS activity results. The molecular basis of this change is not known (Deuel and Prusiner, 1974). These results emphasise the very central role that glutamine plays in controlling its own synthesis.

Adenylylation has been demonstrated in E. coli, S. typhimurium, Shigella flexneri, Klebsiella spp. Pseudomonas putida and recently some phototrophic bacteria, whereas the GSs of several Gram-positive organisms like B. subtilis, B. licheniformis, S. citrea, L. plantarum are not regulated by adenylylation (Gancedo and Holzer, 1968). It has therefore been suggested that regulation by adenylylation is the exclusive property of Gram-negative bacteria (Shapiro and Stadtman, 1970). At first glance, the non-involvement of adenylylation in regulation of Bacillus spp. GSs seems to indicate that glutamine metabolism in Bacillus is not as finely controlled as it is in E. coli. This, however, is not necessarily the case. As noted above, glutamine ultimately seems to be the effector that senses the state of nitrogen nutrition and modulates the rate of glutamine metabolism accordingly, in both E. coli and Bacillus species. In microorganisms, repression of enzyme synthesis by NH_4^+ or glutamine or feedback inhibition, observed in Lactobacillus arabinosus and B. subtilis are sufficient mechanisms for regulation of GS (Schutt and Holzer, 1972).

It would be interesting to identify the nitrogen repressor metabolite (which seems to be glutamine) and assess its

distribution under conditions of nitrogen repression and derepression. Cloned structural and regulatory genes of the nitrogen-circuit can provide the DNA probes needed to directly examine regulation of transcription and translation in vivo and to eventually develop an in vitro system with which to study regulation with purified components.

The addition of glucose to actively growing cultures of V. alginolyticus stimulated GS_T activity. This effect was not dependent on the nitrogen source nor was it a function of the growth rate. Glucose-enhancement of GS occurred even in cultures grown in ASMM (7.7 mM). The glucose-dependent increase is not due to increased growth rate as the enzyme activity is calculated as a function of cell density. In addition, it was of interest that there was little or no difference at all in the growth rate with or without glucose. The addition of cyclic AMP had no effect on this enhancement phenomenon. Phibbs and Bernlohr (1971) reported that repression of GDH synthesis during growth on L-glutamate or casamino acids was overcome by additions of glucose or pyruvate. They found that addition of glucose to exponential-phase cells resulted in a rapid increase of GDH to very high levels. They reported that glucose lengthened the period of exponential growth but that there was no effect on the specific growth rate. The data was interpreted to suggest that the synthesis of GDH may be regulated by some metabolite of glucose even in the presence of large amounts of its reaction product (glutamate).

The stimulatory effect on levels of GS_T activity observed in V. alginolyticus was not restricted merely to glucose. The effect was not sugar-specific and high GS levels were obtained with a variety of sugars. The highest level of GS_T activity was obtained with the addition of sucrose. Arabinose and lactose repressed GS_T activity. V. alginolyticus was not able to grow on these two sugars as sole carbon sources so the lack of a stimulatory effect was not surprising. However, for the same reason a repressive effect would also not be expected. The stimulatory effect of sucrose, fructose, glycerol, glucose and maltose on GS_T activity and the ability of V. alginolyticus to utilize these sugars suggests that a degradation product or products of these sugars may be the actual stimulator.

Long et al. (1981) studied the effect of different carbon sources on alkaline protease activity in V. alginolyticus. Interestingly, they reported repression of protease activity by glucose, sucrose, fructose, glycerol and maltose but no effect with lactose or arabinose, which is reasonable as they are not utilized. Growth of V. alginolyticus on different carbon sources had no effect on histidase and urocanase activities (M.A. Mothibeli, personal communication). Only glucose repressed both histidase and urocanase activity, which confirms results presented in this thesis. Reid et al. (1978) reported repression of collagenase synthesis with glucose, fructose, sucrose, maltose, glycerol and pyruvic acid; arabinose and lactose inhibited collagenase production by 50 and 30% respectively.

3.4.6. THE GDH AND GOGAT ENZYMES IN V. ALGINOLYTICUS

The levels of GDH and GOGAT from V. alginolyticus cells grown in the presence of high and low $(NH_4)_2SO_4$ concentrations and glutamate (50 mM) were investigated. Formation of GDH is repressed in V. alginolyticus cells grown under conditions of nitrogen limitation. This resembles the situation in K. aerogenes (Meers et al., 1970) and K. pneumoniae (Tyler, 1978) but not in E. coli (Senior, 1975) or S. typhimurium (Brenchley et al., 1975). E. coli uses GDH to incorporate ammonia during nitrogen limitation. The GDH levels of S. typhimurium do not decrease when glutamine synthetase is derepressed during growth with limiting ammonia or glutamate (Brenchley et al., 1975).

Cell-free extracts of B. licheniformis and B. cereus contained markedly increased levels of NADP-dependent GDH when NH_4^+ was the sole nitrogen source and low levels in the presence of glutamate (Phibbs and Bernlohr, 1971). NAD-dependent GDH was not present in five species of Bacillus irrespective of the nutritional conditions (Phibbs and Bernlohr, 1971). Most bacteria contain only 1 species of GDH enzyme. E. coli also contains only a NADP-dependent enzyme. GDH occupies a strategic position in nitrogen metabolism and is an important branch point between nitrogen and carbon metabolism, since it catalyses either the reductive amination of α-KG to yield glutamate or the oxidative deamination of glutamate which provides ammonia. GDH seems to have a biosynthetic function as growth of V. alginolyticus in the presence of glutamate

results in low levels of the enzyme. In contrast to *E. coli*, in *K. aerogenes* and now *V. alginolyticus*, GDH is repressed during nitrogen limitation, whereas GOGAT is induced. Senior (1975) suggests that the absence of close linkage in repression and induction between GS and GOGAT in *E. coli* may be due to there being no close genetic linkage between the structural genes of *E. coli* K-12 coding for GS, GDH and GOGAT. The situation in *V. alginolyticus* appears to differ markedly from that in *E. coli*; there seems to be a clear reciprocal relationship between the activity of GS and GDH in *V. alginolyticus* as in *K. aerogenes* (Magasanik *et al.*, 1973).

GOGAT is part of a physiological cycle in which its product glutamate is converted to its substrate glutamine. Considering these characteristics, the function and control of GOGAT is likely to be more complicated than anticipated (Dendinger *et al.*, 1980). Relatively little is known about the regulation of formation of GOGAT. Under conditions of ammonia limitation, *V. alginolyticus* cells produced high levels of GOGAT. This is consistent with the suggestion of Meers *et al.* (1970) that GOGAT is necessary for ammonia assimilation under conditions where the extracellular ammonia concentration is low. Growth of *V. alginolyticus* under conditions of high concentrations of either NH_4^+ or glutamate resulted in low levels of GOGAT. Cells of either *K. aerogenes* or *E. coli* grown with glutamate as the sole nitrogen source contained low levels of GOGAT (Meers *et al.*, 1970; Senior, 1975).

It will be interesting to test what effect glucose has on GDH synthesis under conditions of ammonia-excess. Under conditions of nitrogen limitation, GOGAT was subject to repression by glucose. The effect of glucose on the ammonia assimilatory enzymes is certainly unusual; it repressed GOGAT production in the conventional manner but caused enhancement of GS_T activity, regardless of the nitrogen source present in the growth medium. Extrapolating from this observation, it can be assumed that glucose does not enhance GDH levels.

The likelihood of factors other than ammonia availability being involved in the repression and derepression of GS is certainly suggested by these studies. The level of GS seems to be rather a complex function of both carbon and nitrogen sources. These data clearly indicate the relative roles of the two pathways for ammonia assimilation. Some data (Brenchley et al., 1975; Senior, 1975) has even shown an inverse relationship between GS and GOGAT levels. In V. alginolyticus, however, both GS and GOGAT are higher in nitrogen-limited cultures than in those with excess NH_4^+. When V. aliginolyticus cells are grown in the presence of excess ammonia, glutamate is formed by way of GDH, which does not require a large investment of energy as does the coupled GS-GOGAT pathway. Under these conditions, relatively low GS and GOGAT activities suffice to provide the glutamate and glutamine required for protein synthesis and for biosynthetic reactions. However, when the available supply of ammonia is limited, the GDH reaction does not function efficiently

and ammonia is assimilated by the GS-GOGAT pathway.

Further work involving similar growth and activity studies in mutants lacking one or more of the three ammonia assimilatory enzymes could prove fruitful. By these studies, the regulatory functions controlling the cell's responses to these deficiencies could be elucidated and the individual contributions of each enzyme to nitrogen assimilation could therefore be assessed.

CHAPTER 4

CONCLUSIONS

The aim of this work was to study the regulation of nitrogen metabolism in V. alginolyticus. Studies on collagenase and protease production in V. alginolyticus implicated the involvement of the hut enzymes and GS (Reid et al., 1978; Long et al., 1981). The mechanisms governing the regulation of the inducible nitrogen catabolic enzymes and the ammonia assimilatory enzymes in V. alginolyticus were investigated.

Control of enzymes of nitrogen metabolism at the transcriptional level involves both induction and nitrogen catabolite repression. Although the hut enzymes were coordinately induced by histidine, there were differences in their regulation by nitrogen-containing compounds. This suggests that although the hut enzymes of V. alginolyticus have common elements of control, they are situated in different operons. Histidase, specifically induced by histidine, was subject to control by temperature and oxygen. The production of collagenase and the serine proteases was affected by temperature, oxygen and histidine at the level of transcription (Hare et al., 1981). Glutamine caused severe repression of the levels of collagenase, protease, histidase urocanase and GS in V. alginolyticus. Cyclic AMP did not relieve glucose repression of histidase, collagenase and protease. These enzymes were not regulated by "classical" catabolite repression and the role of cyclic AMP needs further investigation.

The addition of $(NH_4)_2SO_4$ stimulated the production of histidase, arginase and GDH and had no effect on the synthesis of alanine dehydrogenase; but repressed the production of urocanase, FGA-hydrolase, collagenase, protease, GOGAT and GS. Growth of V. alginolyticus in various amino acids stimulated histidase and GS activity. The GS of C. aurantiacus was stimulated by growth in amino acids and ammonium ions (Kaulen and Klemme, 1983). Since this study has shown that the "classical" nitrogen catabolite repression control system does not regulate the production of histidase, arginase and alanine dehydrogenase in V. alginolyticus, some other mechanism for linking nitrogen source and growth rate with metabolism must be proposed. In order to understand the regulation of nitrogen metabolism in V. alginolyticus, the genetic analysis of various structural and control mutants is necessary.

The involvement of some aspect of carbon metabolism seems likely in the regulation of GS since growth in various carbon sources resulted in the stimulation of GS activity. This is an unusual phenomenon and requires further investigation. Not much attention has been given to studies on the effect of carbon source on the enzymes involved in nitrogen metabolism. Phibbs and Bernlohr (1971) suggested that the glucose-dependent increase in GDH activity in B. licheniformis was regulated by induction by some metabolite of glucose (Section 3.4.5). Studies on the effect of pyruvic acid on GS activity in V. alginolyticus must be carried out to establish whether it is responsible for the stimulation of GS production. All

the sugars may mediate their stimulatory effect via pyruvic acid. Since arabinose and lactose cannot be utilized by V. alginolyticus, repression of GS must be by virtue of these sugars themselves. The effect of these sugars on the production of GDH and GOGAT need to be investigated. Protease production in V. alginolyticus was repressed by most carbon sources with the exception of lactose and arabinose (Long et al., 1981). This result is in direct contrast to the situation observed with GS. This is reinforced by data obtained from growth of V. alginolyticus in various amino acids. Protease production was repressed by most amino acids with the exception of histidine, while GS production was enhanced by various amino acids. Collagenase production was repressed by various carbon sources and amino acids (Reid, 1981). Since these enzymes are all involved in nitrogen metabolism, their regulation would be expected to be similar. It is therefore interesting that the situation seems to be the opposite. The techniques for the establishment of a V. alginolyticus gene bank in E. coli have been developed and a gene library obtained. Future work will involve determining whether the regulation of these systems is affected or altered in another Gram-negative cell. Of special interest in future work is the investigation of the mechanisms involved in the stimulation of histidase activity by ammonium ions and the increase in GS production by growth of V. alginolyticus in various carbon sources.

GS in V. alginolyticus is subject to repression and derepression as a function of nitrogen and carbon source present in

the medium, buffer preference, divalent cation concentration, feedback inhibition that is antagonistic, and an adenylylation-deadenylylation system. The main route of ammonia assimilation in V. alginolyticus under conditions of nitrogen limitation is the coupled GS-GOGAT pathway while under conditions of nitrogen excess the GDH pathway is operative. The elucidation of the basic characteristics and regulatory properties of GS have been presented in this thesis. In conjunction with this study, the recent development of a method of purification of this enzyme allows for a more detailed inquiry into the complexities of its catalytic and regulatory mechanisms.

This study indicates that the regulation of nitrogen metabolism in V. alginolyticus resembles regulation in both the Gram-negative and Gram-positive microorganisms. The GS of V. alginolyticus is regulated by an adenylylation-deadenylylation system (P. Brandt, pers. comm.) which is typical of all Gram-negative bacteria. NH_4^+-shocking does not affect the activity of GS in B. subtilis which is consistent with the lack of an adenylylation system. The GS of V. alginolyticus is subject to severe repression by glutamine which is a characteristic of Gram-positive bacteria (Section 3.4.5). Regulation of V. alginolyticus resembles Gram-positive Bacillus strains in that both groups produce true extracellular proteases during the stationary growth phase (Welton and Woods, 1973; Welton and Woods, 1975; Priest, 1977; Long et al., 1981); protease production involves stable mRNA (Both et al., 1972; O'Connor et al., 1978; Reid et al., 1980).

Catabolite repression is not relieved by cyclic AMP (Priest, 1977; Long *et al.*, 1981; Glenn, 1976; Reid *et al.*, 1978), histidine is the inducer of the *hut* enzymes (Chasin and Magasanik, 1968; Bowden *et al.*, 1982), and the inducible nitrogen catabolic enzymes, arginase, alanine dehydrogenase and histidase are not regulated by nitrogen catabolite repression (Schreier *et al.*, 1982), and severe repression of GS by glutamine (Hubbard and Stadtman, 1967). The activity of GS in *E. coli* and *Bacillus* spp. varies as a function of nitrogen source (Shapiro and Stadtman, 1970). GS synthesis is repressed under conditions of nitrogen excess and derepressed when nitrogen is limiting. GSs from Gram-positive and Gram-negative organisms are effectively inhibited by mixtures of end-products from the diverse pathways of glutamine metabolism (Shapiro and Stadtman, 1970). Unlike K. aerogenes (Dendinger *et al.*, 1980), B. licheniformis (Schreier *et al.*, 1982) and *V. alginolyticus*, *E. coli* (Senior, 1975) and *S. typhimurium* (Brenchley *et al.*, 1975) use GDH to incorporate ammonia during nitrogen limitation. GDH in *V. alginolyticus* appears to have a biosynthetic function since growth on glutamate represses the activity of this enzyme. This resembles the situation in the *Bacillus* spp. (Phibbs and Bernlohr, 1971) and in *E. coli* (Tyler, 1978) but not in *S. typhimurium* (Brenchley *et al.*, 1975).

In *K. aerogenes*, the *Bacillus* spp., *Pseudomonas fluorescens* and *V. alginolyticus*, conditions that favoured the synthesis of GOGAT generally favoured synthesis of GS and caused suppression of GDH formation (Meers *et al.*, 1970). No simple

model for the co-ordinated control of GDH and GOGAT syntheses can be suggested (Meers et al., 1970). Cells of K. aerogenes and E. coli also contained repressed levels of GOGAT when grown in glutamate (Meers et al., 1970; Senior, 1975).

A number of regulatory principles have emerged from this study. A unifying concept propounded by Shapiro and Stadtman (1970) seems to hold true: "the regulatory function is more constant than the regulatory mechanism." Thus, the same principle is not always used for controlling comparable functions in one organism, nor for controlling identical functions in different organisms.

APPENDIX

MEDIA

Complex medium agar

Casein hydrolysate	17.0	g
NaCl	23.4	g
glycerol	10.0	g
Na_2SO_3	0.1	g
nutrient broth	8.0	g
soytone	3.0	g
tryptone	0.5	g
vitamin free Casamino Acids	0.5	g
yeast extract	2.0	g
glucose	5.0	g
Difco agar	15.0	g
distilled water	1000	ml

Adjust the pH to 7.6 with NaOH.

Ammonia succinate minimal medium (ASMM)

Salt solution: K_2HPO_4	10.6	g
KH_2PO_4	4.56	g
sodium citrate	0.48	g
$(NH_4)_2SO_4$	1.0	g
$MgSO_4.7H_2O$	0.1	g
(added cold in solution)		

Saline solution:	NaCl	23.4 g
	distilled water	888 ml

Sodium succinate solution: 25% (W/V) in distilled water

To make up:	Salt solution	100 ml
	$MgSO_4$	2 ml
	Saline solution	888 ml
	Sodium succinate	10 ml

10mM Imidazole - HCl Buffer + 2.5mM $MgCl_2$

Imidazole buffer substance	0.68 g
$MgCl_2$	0.51 g
distilled water	1000 ml

Adjust the pH to 7.15 with concentrated HCl.

0.5M Potassium Phosphate Buffer (pH 7.4)

Solution A :	0.5M KH_2PO_4	6.8 g/100 ml
Solution B :	0.5M K_2HPO_4	17.42 g/200 ml

For pH 7.4	Solution A :	38 ml
	Solution B :	162 ml

Tris - HCl Buffer

NaCl	23.4 g
$CaCl_2.2H_2O$	0.29 g
Tris (0.1M)	12.10 g
distilled water	1000 ml

Adjust the pH to 7.6 with concentrated HCl.

L I T E R A T U R E C I T E D

ALEF, K., and W. G. ZUMFT. 1981. Regulatory properties of glutamine synthetase from the nitrogen-fixing phototrophic bacterium Rhodopseudomomas palustris. Z. Naturforsch 36c : 784 - 789.

BACKMAN, K.C., Y.M. CHEN, S. UENO-NISHIO, and B. MAGASANIK. 1983. The product of gln L is not essential for regulation of bacterial nitrogen assimilation. 1983. J. Bacteriol. 154 : 516 - 519.

BAUMANN, L., and P. BAUMANN. 1980. Immunological relationships of glutamine synthetases from marine and terrestrial enterobacteria. Curr. Microbiol. 3 : 191 - 196.

BAUMANN, L., S.S. BANG, and P. BAUMANN. 1980. Study of relationship among species of Vibrio, Photobacterium, and terrestrial enterobacteria by an immunological comparison of glutamine synthetase and superoxide dismutase. Curr. Microbiol. 4 : 133 - 138.

BENDER R.A., K.A. JANSSEN, A.D. RESNICK, M. BLUMENBERG, F. FOOR, and B. MAGASANIK. 1977. Biochemical parameters of glutamine synthetase from Klebsiella aerogenes. J. Bacteriol. 129 : 1001 - 1009.

BENDER R.A., and B. MAGASANIK. 1977. Regulatory mutations in Klebsiella aerogenes structural gene for glutamine synthetase. J. Bacteriol. 132 : 100 - 105.

BENDER R.A., P.M. SNYDER, R. BUENO, M. QUINTO, and B. MAGASANIK. 1983. Nitrogen regulation system of Klebsiella aerogenes : the nac gene. J. Bacteriol. 156 : 444 - 446.

BEUDEKER, R.F., R. RUGMAN, J.G. KUENEN. 1982. Regulation of nitrogen assimilation by the oligate chemolithotroph Thiobacillus neapolitanus. J. Gen. Microbiol. 128 : 39 - 47.

BHANDARI, B., F. VAIRINHOS, and D.J.D. NICHOLAS. 1983. Some properties of glutamine synthetase from Rhizobium japonicum CC705 and CC723. Arch. Microbiol. 136 : 84-88.

BLOOM, F.R., S.L. STREICHER, and B. TYLER. 1977. Regulation of enzyme synthesis by the glutamine synthetase of Salmonella typhimurium : a factor in addition to glutamine synthetase is required for activation of enzyme formation. J. Bacteriol. 130 : 983 - 990.

BOETHLING, R.S. 1975. Regulation of extracellular protease secretion in Pseudomonas maltophilia. J. Bacteriol. 123 : 954 - 961.

BOTH, G.W., J.L. McINNES, J.E. HANLON, B.K. MAY, and W.H. ELLIOT. 1972. Evidence for an accumulation of messenger RNA specific for extracellular protease and its relevance to the mechanism of enzyme secretion in bacteria. J. Mol. Biol. 67 : 199 - 217.

BOWDEN, G., M.A. MOTHIBELI, F.T. ROBB, and D.R. WOODS. 1982. Regulation of hut enzymes and extracellular protease activity in Vibrio alginolyticus hut mutants. J. Gen. Microbiol. 128 : 2041 - 2045.

BRANDT, P.W. 1983. The purification and partial characterization of glutamine synthetase of Vibrio alginolyticus. Hons. Thesis. University of Cape Town, Cape Town, Rep. of South Africa.

BRENCHLEY, J.E., C.A. BAKER, and L.G. PATIL. 1975. Regulation of the ammonia assimilatory enzymes in Salmonella typhimurium. J. Bacteriol. 124 : 182 - 189.

BROMAN, K., N. LAUWERS, V. STALON, and J.M. WIAME. 1978. Oxygen and nitrate utilization by Bacillus licheniformis of the arginase and arginine deiminase routes of arginine catabolism and other factors affecting their syntheses. J. Bacteriol. 135 : 920 - 927.

CHASIN, L.A., and B. MAGASANIK. 1968. Induction and repression of the histidine-degrading enzymes of Bacillus subtilis. J. Biol. Chem. 243 : 5165 - 5178.

DAVIES, W., and J.G. ORMEROD. 1982. Glutamine synthetase in Chlorobium limicola and Rhodospirulluim rubrum. FEMS Microbiol. Letts. 13 : 75 - 78.

de BRUIJN, F.J., and F.M. AUSUBEL. 1983. The cloning and characterization of the gln F (ntr A) gene of Klebsiella pneumoniae : Role of gln F (ntr A) in the regulation of nitrogen fixation (nif) and other nitrogen assimilation genes. Mol. Gen. Genet. 192 : 342 - 353.

DE CROMBRUGGHE, B., R.L. PERLMAN, H.E. VARMUS, and I. PASTAN. 1969. Regulation of inducible enzyme synthesis in Escherichia coli by cyclic adenosine 3',5' - monophosphate. J. Biol. Chem. 244 : 5828 - 5835.

DENDINGER, S.M., L.G. PATIL, and J.E. BRENCHLEY. 1980. Salmonella typhimurium mutants with altered glutamate dehydrogenase and glutamate synthase activities. J. Bacteriol. 14 : 190 - 198.

DEUEL, T.F., and E.R. STADTMAN. 1970. Some kinetic properties of Bacillus subtilis glutamine synthetase. J. Biol. Chem. 245 : 5206 - 5213.

DEUEL, T.F., A. LERNER, and D. ALBRYCHT. 1973. Regulation of glutamine synthetase from rat liver and rat kidney. p 129-144. In The enzymes of glutamine metabolism, eds. S. Pruisner and E.R. Stadtman. Academic Press, New York and London.

DEUEL, T.F., and S. PRUSINER. 1974. Regulation of glutamine synthetase from Bacillus subtilis by divalent cations, feedback inhibitions and L-glutamine. J. Biol. Chem. 249 : 257 - 264.

DONOHUE, T.J., and R.W. BERNLOHR. 1981. Regulation of the activity of the Bacillus licheniformis A5 glutamine synthetase. J. Bacteriol. 148 : 174 - 182.

EADY, R.R., R. ISSACK, C. KENNEDY, J.R. POSTGATE, and H.D. RATCLIFFE. 1978. Nitrogenase synthesis in Klebsiella pneumoniae: Comparison of ammonium and oxygen regulation. J. Gen. Microbiol. 104 : 277 - 285.

FALK, G., B.C. JOHANSSON, and S. NORDLUND. 1982. The role of glutamine synthetase in the regulation of nitrogenase activity ("switch off" effect) in Rhodospirullum rubrum. Arch. Microbiol. 132 : 251 - 253.

FOOR, F., K.A. JANSSEN, and B. MAGASANIK. 1975. Regulation of the synthesis of glutamine synthetase by adenylylated glutamine synthetase. Proc. Natl. Acad. Sci. USA. 77 : 2636 - 2640.

GAILLARDIN, M.C., and B. MAGASANIK. 1978. Involvement of the product of the gln F gene in the autogenous regulation of glutamine synthetase formation in Klebsiella aerogenes. J. Bacteriol. 133 : 1329 - 1338.

GANCEDO, G., and H. HOLZER. 1968. Enzymatic inactivation of glutamine synthetase in enterobacteriaceae. Eur. J. Biochem. 4 : 190 - 192.

GINSBURG, A., and E.R. STADTMAN. 1973. Regulation of glutamine synthetase in Escherichia coli , p9-43. In The Enzymes of glutamine metabolism, eds. S. Prusiner and E.R. Stadtman. Academic Press, New York and London.

GLENN, A.R. 1976. Production of extracellular proteins by bacteria. Annu. Rev. Microbiol. 30 : 41 - 62.

GOLDBERG, R.B., and R. HANAU. 1980. Regulation of Klebsiella pneumoniae hut operons by oxygen. J. Bacteriol. 141 : 745 - 750.

HARE, P., S. LONG, F.T. ROBB, and D.R. WOODS. 1981. Regulation of exoprotease production by temperature and oxygen in Vibrio alginolyticus. Arch. Microbiol. 130 : 276 - 280.

HARTWELL, L.H., and B. MAGASANIK. 1963. The Molecular basis of histidase induction in Bacillus subtilis. J. Mol. Biol. 7 : 401 - 420.

HENNECKE, H., and K.T. SHANMUGAN. 1979. Temperature control of nitrogen fixation in Klebsiella pneumoniae. Arch. Microbiol. 123 : 259 - 265.

HILL, S., C. KENNEDY, E. KAVANAGH, R.B. GOLDBERG, and R. HANAU. 1981. Nitrogen fixation gene (nif L) involved in oxygen regulation of nitrogenase synthesis in Klebsiella pneumoniae. Nature 290 : 424 - 426.

HUBBARD, J.S., and E.R. STADTMAN. 1967. Regulation of glutamine synthetase. V. Partial purification and properties of glutamine synthetase from Bacillus licheniformis. J. Bacteriol. 94 : 1007 - 1015.

HUBBARD, J.S., and E.R. STADTMAN. 1967 a. Regulation of glutamine synthesis. II. Patterns of feedback inhibition in microorganisms. J. Bacteriol. 93 : 1045 - 1055.

KAULEN, H., and J.H. KLEMME. 1983. No evidence of covalent modification of glutamine synthetase in the thermophilic phototrophic bacterium Chloroflexus aurantiacus. FEMS Microbiol. Letts. 20 : 75 - 79.

KENDRIK, K.E., and M.L. WHEELIS. 1982. Histidine dissimilation in Streptomyces coelicolor. J. Gen. Microbiol. 128 : 2029 - 2040.

KLEINSCHMIDT, J.A., & D. KLEINER. 1978. The glutamine synthetase from Azotobacter vinelandii : purification, characterization, regulation and localization. Eur. J. Biochem. 89 : 51 - 60.

KUSTU, S., D. BURTON, E. GARCIA, L. McCARTER, N. McFARLAND. 1979. Nitrogen control in Salmonella : regulation by the gln R and gln F gene products. Proc. Natl. Acad. Sci. USA. 76 : 4576 - 4580.

LEPO, J.E., O. WYSS, and F.R. TABITA. 1982. Regulation and biochemical characterization of the glutamine synthetase of Azotobacter vinelandii. Biochim. Biophys. Acta. 704 : 414 - 421.

LONG, S., M.A. MOTHIBELI, F.T. ROBB, and D.R. WOODS. 1981. Regulation of extracellular alkaline protease activity by histidine in a collagenolytic Vibrio alginolyticus. J. Gen. Microbiol. 127 : 193 - 199.

LUND, P., and B. MAGASANIK. 1965. N-formimino-L-glutarate-formimino hydrolase of Aerobacter aerogenes. J. Biol. Chem. 240 : 4316 - 4319.

MAGASANIK, B. 1982. Genetic control of nitrogen assimilation in bacteria. Ann. Rev. Genet. 16 : 135 - 168.

MAY, B.K., and W.H. ELLIOTT. 1968. Characteristics of extracellular protease formation by Bacillus subtilis and its control by amino aud repression. Biochim. Biophys. Acta. 157 : 607 - 615.

McCOWEN, S.M., and P.V. PHIBBS, JR. 1974. Regulation of alanine dehydrogenase in Bacillus licheniformis. J. Bacteriol. 118 : 590 - 597.

McFARLAND, N., L. McCARTER, S. ARTZ, S. KUSTU. 1981. Nitrogen regulatory locus "gln R" of enteric bacteria is composed of cistrons ntr B and ntr C : identification of their products. Proc. Natl. Acad. Sci. USA. 78 : 2135 - 2159.

MEERS, J.L., D.W. TEMPEST, and C.M. BROWN. 1970. "Glutamine (amide) : 2 - oxoglutarate amino transferase oxido - reductase (NADP)", an enzyme involved in the synthesis of glutamate by some bacteria.

MERRICK, M.J. 1982. A new model for nitrogen control. Nature. 279 : 362 - 363.

MERRICK, M.J., S. HILL, H. HENNECKE, M. HAHN, R. DIXON, and C. KENNEDY. 1982. Repressor properties of the nif L gene product in Klebsiella pneumoniae. Mol. Gen. Genet. 185 : 75 - 81.

O'CONNOR, R., W.H. ELLIOT, and B.K. MAY. 1978. Modulation of an apparent mRNA pool for extracellular protease in Bacillus amyloliquefaciens. J. Bacteriol. 136 : 24-34.

OW, D.W., V. SUNDARESAN, D.M. ROTHSTEIN, S.E. BROWN, and F.M. AUSUBEL. 1983. Promoters regulated by the gln G (ntr C) and nif A gene products share a heptameric consensus sequence in the -15 region. Proc. Natl. Acad. Sci. USA. 80 : 2524 - 2528.

PAHEL, G., and B. TYLER. 1979. A new gln A-linked regulatory gene for glutamine synthetase in Escherichia Coli. Proc. Natl. Acad. Sci. USA. 76 : 4544 - 4548.

PAHEL, G., D.M. ROTHSTEIN, and B. MAGASANIK. 1982. Complex gln A-gln L - gln G operon of Escherichia coli. J. Bacteriol. 150 : 202-213.

PAN, F.L., and J.G. COOTE. 1979. Short communications. Glutamine synthetase and glutamate synthase activities during growth and sporulation in Bacillus subtilis. J. Gen. Microbiol. 112 : 373 - 377.

PHIBBS JR, P.V., and R.W. BERNLOHR. 1971. Purification, properties and regulation of glutamic dehydrogenase of Bacillus licheniformis. J. Bacteriol. 106 : 375 - 385.

PRIEST, F.G. 1977. Extracellular enzyme synthesis in the genus Bacillus. Bacteriol. Rev. 41 : 711 - 753.

PRIVAL, M.J., J.E. BRENCHLEY, and B. MAGASANIK. 1973. Glutamine synthetase and the regulation of histidase formation in Klebsiella aerogenes. J. Biol. Chem. 248 : 4334 - 4344.

PRIVAL, M.J., and B. MAGASANIK. 1971. Resistance to catabolite repression of histidase and proline oxidase during nitrogen-limited growth of Klebsiella aerogenes. J. Biol. Chem. 245 : 6288 - 6296.

RATNER, S. 1962. Transamidinase. Methods Enzmol. 5 : 843 - 848.

RAVEL, J.M., J.S. HUMPHREYS, and W. SHIVE. 1965. Control of glutamine synthesis in Lactobacillus arabinosus. Arch. Biochem. Biophys. 111 : 720 - 726.

REID, G.C. 1981. Studies on the regulation of extracellular collagenase production by Vibrio alginolyticus. Ph.D. Thesis. University of Cape Town, Cape Town, Rep. of South Africa.

REID, G.C., F.T. ROBB, and D.R. WOODS. 1978. Regulation of extracellular collagenase production in Achromobacter iophagus. J. Gen. Microbiol. 109 : 149 - 154.

REID, G.C., D.R. WOODS, and F.T. ROBB. 1980. Peptone induction and rifampicin - insensitive collagenase production by Vibrio alginolyticus. J. Bacteriol. 142 : 447 - 454.

REYSSET, G. 1981. New class of Bacillus subtilis glutamine-requiring mutants. J. Bacteriol. 148 : 653-658.

ROTHMAN, N., D. ROTHMAN, F. FOOR, and B. MAGASANIK. 1982. Role of gln A-linked genes in regulation of glutamine synthetase and histidase formation in Klebsiella aerogenes. J. Bacteriol. 150 : 221 - 230.

SCHREIER, H.J., T.M. SMITH, and R.W. BERNLOHR. 1982. Regulation of nitrogen-catabolic enzymes in Bacillus spp. J. Bacteriol. 15 : 971 - 975.

SCHUTT, H., and H. HOLZER. 1972. Biological function of the ammonia-induced inactivation of glutamine synthetase in Escherichia coli. Eur. J. Biochem. 26 : 68 - 72.

SENIOR, P.J. 1975. Regulation of nitrogen metabolism in Escherichia coli and Klebsiella aerogenes : studies with continuous-culture technique. J. Bacteriol. 123 : 407 - 418.

SHAPIRO, B.M., and E.R. STADTMAN. 1970. The Regulation of glutamine synthesis in microorganisms. Annu. Rev. Microbiol. 24 : 501 - 524.

SMITH, G.R., J.S. HALPERN, and B. MAGASANIK. 1971. Genetic and metabolic control of enzymes responsible for histidine degradation in Salmonella typhimurium. J. Biol. Chem. 246 : 3320 - 3329.

STADTMAN, E.R., A. GINSBURG, J.E. CIARDI, S. YEH, B. HENNIG, and B.M. SHAPIRO. 1970. Multiple molecular forms of glutamine synthetase produced by enzyme catalyzed adenylylation and deadenylylation reactions. Adv. Enzy. Regul. 8 : 99 - 118.

STADTMAN, E.R., and A. GINSBURG. 1974. The glutamine synthetase of Escherichia coli : Structure & control. p755-807. In The Enzymes. Vol. 10. Academic Press, New York.

STINSON, M.W., and J.M. MERRICK. 1974. Extracellular enzyme secretion by Pseudomonas lemoignei. J. Bacteriol. 119 : 152 - 161.

ST. JOHN, R.T., V.K. SHAH, and W.J. BRILL. 1974. Regulation of nitrogenase synthesis by oxygen in Klebsiella pneumoniae. J. Bacteriol. 119 : 266 - 269.

STREICHER, S.L., and B. TYLER. 1976. New methods for the purification of glutamine synthetase (GS) from Klebsiella aerogenes indicate the presence of a GS-DNA complex in the bacterial cells. Abstract. Fed. Proc. 35 : 1471.

TANAKA, S., and S. IUCHI. 1971. Induction and repression of an extracellular proteinase in Vibrio parahaemolyticus. Biken. J. 14 : 81 - 96.

TEMPEST, D.W., J.L. MEERS, and C.M. BROWN. 1970. Short communications. Synthesis of glutamate in Aerobacter aerogenes by a hitherto unknown route.

TEMPEST, D.W., J.L. MEERS, and C.M. BROWN. 1973. Glutamate synthetase (GOGAT); a key enzyme in the assimilation of ammonia by prokaryotic organisms, p167-182. In The Enzymes of glutamine metabolism, eds. S. Prusiner and E.R. Stadtman. Academic Press, New York and London.

TULI, R., R. FISHER, and R. HASELKORN. 1982. The ntr genes of Escherichia coli activate the hut and nif operons of Klebsiella pneumoniae. Gene 19 : 109 - 116.

TYLER, B. 1978. Regulation of the assimilation of nitrogen compounds. Annu. Rev. Biochem. 47 : 1127 - 1162.

TYLER, B., A.B. DELEO, and B. MAGASANIK. 1974. Activation of transcription of hut DNA by glutamine synthetase. Proc. Natl. Acad. Sci. USA. 71 : 225 - 229.

UENO-NISHIO, S., K.C. BACKMAN, and B. MAGASANIK. 1983. Regulation at the gln L-operator - promoter of the complex gln ALG operon of Escherichia coli. J. Bacteriol. 153 : 1247 - 1251.

WEDLER, F.C., J. CARFI, and A.E. ASHOUR. 1976. Glutamine synthetase of Bacillus stearothermophilus. Regulation, site, interactions, and functional information. Biochemistry 15 : 1749 - 1755.

WELTON, R.L., and D.R. WOODS. 1973. Halotolerant collagenolytic activity of Achromobacter iophagus. J. Gen. Microbiol. 75 : 191 - 196.

WELTON, R.L., and D.R. WOODS. 1975. Collagenase production by Achromobacter iophagus. Biochim. Biophys. Acta. 384 : 228 - 234.

WOHLHUETER, R.M., H. SCHUTT, and H. HOLZER. 1973. Regulation of glutamine synthesis in vivo in E.coli^{+}. p45-64. In The enzymes of glutamine metabolism, eds. S. Pruisner and E.R. Stadtman. Academic Press, New York and London.

WOOLFOLK, C.A., and E.R. STADTMAN. 1964. Cumulative feedback inhibition in the multiple end product regulation of glutamine synthetase activity in Escherichia coli. Biochem. Biophys. Res. Commun. 17 : 313 - 319.

WOOLFOLK, C.A., and E.R. STADTMAN. 1967. Regulation of glutamine synthetase III. Cumulative feedback inhibition of glutamine synthetase from Escherichia coli. Arch. Biochem. Biophys. 118 : 736 - 755.

YOCH, D.C., M. CANTU, and Z.M. ZHANG. 1983. Evidence for a glutamine synthetase-chromatophore association in the phototroph Rhodospirullum rubrum : Purification, properties, and regulation of the enzyme. J. Bacteriol. 154 : 632 - 639.

YOSHIKAWA, M., F. MATSUDA, M. NAKA, E. MUROFUSHI, and Y. TSUNEMATSU. 1974. Pleiotropic alterations of activities of several toxins and enzymes in mutants of Staphylococcus aureus. J. Bacteriol. 119 : 117 - 122.