CONCLUSION
REVIEW OF LITERATURE

Glutamine synthetase is one of the key enzymes for ammonia assimilation in a variety of plant and bacterial systems (Shapiro and Stadtman, 1970b; Tyler, 1978; Miflin and Lea, 1976; Miflin, 1983). The enzyme from E. coli, an enteric, coliform, gram negative bacteria has been exhaustively characterized and has been found to be regulated by a complex set of mechanisms (Magasanik et al, 1974) involving feedback inhibition, repression and adenylylation/deadenylylation (Ginsburg and Stadtman, 1973; Wolhueter, 1973). Regulation of GS by feedback inhibition has been extensively discussed in the previous chapter. This chapter deals with the adenylylation/deadenylylation control mechanism of regulation and its occurrence in biological systems.

The mechanism of activation (deadenylylation) and inactivation (adenylylation) of GS from E. coli has been elucidated by Stadtman and his co-workers (Ginsburg and Stadtman, 1973). E. coli GS is a dodecamer with a molecular weight of approximately 600,000d. The enzyme is inactivated by the attachment of adenylyl groups, to a maximum of 12, one to a tyrosine residue on each of the subunits. Adenylylation utilizes ATP and requires two proteins called adenylyl transferase (ATase) and PII.

\[ \text{INACTIVATION OF GS.} \]
These same proteins are also required for the removal of the adenylyl groups (activation) which restores the biosynthetic function of GS.

Whether ATase and张11 catalyze adenylylation or deadenylylation depends on the form of张11. The attachment of uridylyl groups to this protein converts it from张11 form (which activates adenylylation) to张11,UMP (which activates deadenylylation). The uridylylation of张11 is catalyzed by an enzyme called uridylyl transferase (UTase) and the removal of UMP from张11 is catalyzed by another enzyme called uridylyl removing enzyme (URenz). The activity of UTase and张11 are regulated by levels of ATP, α-keto glutarate, glutamine and divalent metal ions (shown as positive (+) and negative (-) effectors in the closed circles in the diagrammatic sketch).

The adenylylation state of GS governs the effects of feedback modifiers and divalent cations on enzyme activity thus forming a fine control mechanism of GS regulation. Another effect of adenylylation is
to ensure that glutamine inhibits its own biosynthesis (by
indirectly affecting GS through the enzymes of the adenylylation
cascade). This system of control is well documented in gram negative
bacteria.

In gram positive bacteria, however, it has been found to be
absent and alternative mechanisms for regulating GS have been
described (Deuel and Prusiner, 1974; Wedler, 1974b; Wedler and
Hoffmann, 1974b; Wedler et al., 1980). Thus in the absence of
adenylylation, glutamine effects enzyme activity directly and, in
combination with other effectors like AMP can cause synergistic
patterns of inhibition.

Nitrogen fixing cyanobacteria also appear to be lacking the
covalent modification system (Dharamawardene et al., 1973; Rowell et al.,
1977; Stacey et al., 1979). Not much is known about the regulatory
systems operative for the control of GS in non-nitrogen fixing
cyanobacteria (Emond et al., 1979).

In an attempt to understand the regulation of GS in the non-
nitrogen fixing, protein rich cyanobacterium Spirulina platensis, the
presence of adenylylation/deadenylylation control mechanism was checked.
In the process a novel situation, whereby Mg\textsuperscript{++} consistently caused
enhancement of Mn\textsuperscript{++}-dependent reverse transferase activity, was
observed. This system had no adenylylation/deadenylylation system
of GS regulation.
MATERIALS AND METHODS

Materials

L-alanine, bovine serum albumin, cetyl trimethyl ammonium bromide (CTAB), L-glutamate, L-glutamine, L-serine, hydroxylamine hydrochloride and snake venom phospho-diesterase (SVP) were purchased from Sigma Chemicals Co., U.S.A. All other chemicals used were of analytical grade.

Culture and Growth conditions

Cultures of Spirulina platensis were grown as described in Chapter I. For certain experiments, nitrate was substituted by other specified organic or inorganic nitrogen sources.

Harvesting of Cells and preparation of crude extracts

Cells were harvested as described in Chapter I, with one difference. Prior to harvesting, the cells were treated with CTAB (final concentration in the medium was 0.01%) for one minute. The cells were then harvested by vacuum filtration onto Whatman No. 1 filter papers and washed with four volumes of cold extraction buffer (Tris-HCl, pH 7.4, 0.05 M) containing 1% KCl. The cell mats so obtained were suspended in extraction buffer and disrupted at 4°C using a MSE ultrasonic disintegrator. These cell sonicates served as the crude extracts used for enzyme assays in the experiments unless mentioned otherwise.
Enzyme preparation

A 44-fold purified enzyme sample was prepared as described in Chapter I. This enzyme was used for all kinetics work, unless mentioned otherwise.

Assay Procedure.

GS activity was assayed using the transferase assay described by Shapiro and Stadtman (1970a). Where required, the Mn^{2+} concentration was varied and was replaced or used in combination with other divalent cations, as specified. One enzyme unit is defined as the amount of enzyme required to produce one µ mole of product at 50°C, under optimal assay conditions. Specific activity is expressed as units.mg protein^{-1}. When crude extracts were assayed, the reaction mixtures were clarified by centrifugation before measuring the absorbance at 540 nm.
RESULTS

A preliminary experiment was conducted which utilized the Mn$^{++}$-dependent reverse transferase assay, in the presence and absence of 60 mM Mg$^{++}$, as a measure of the adenylylation state of GS (Table I). With 60 mM Mg$^{++}$, a distinct stimulation in activity was obtained both in crude extracts and in 44 fold purified enzyme preparations (from nitrate grown cultures). Mg$^{++}$, when alone in the reaction mixture, could not account for the enhancement observed when it was present in combination with Mn$^{++}$. The experiment was repeated using crude extracts from cultures grown on a number of organic and inorganic nitrogen sources. Again, in all cases, there was reproducible stimulation caused by Mg$^{++}$ (Table II).

Nitrate grown cells were given a 30 mM ammonium shock in medium, over a period of one hour. This shock in no way affected the enhancement caused by Mg$^{++}$ (Table III).

Snake venom phosphodiesterase treatment of the ammonium shocked cultures, did not affect the Mg$^{++}$ induced enhancement of transferase activity, even after one hour incubation with SVP (Table IV).

The enhancement pattern was found to be specific for Mg$^{++}$, since other divalent cations, like Co$^{++}$, Ni$^{++}$ and Cu$^{++}$, could not replace Mg$^{++}$ and, at 60 mM concentrations, inhibited enzyme activity almost completely (Table V). The stimulation caused by 60 mM Mg$^{++}$ occurred over a concentration range of 0-50 mM Mn$^{++}$. However, the percent enhancement was not constant. Rather, it increased to a maximum at Mn$^{++}$ concentrations that produced optimal enzyme activity and decreased thereafter (Figure 1). Mg$^{++}$-dependent
stimulation occurred over a wide pH range (5.5 to 8.0), but completely disappeared at pH 8.5. The enhancement was nearly constant over the pH range where it occurred (Fig.2).

It was evident from Figure 3, that the divalent cation activator exerted a distinct biphasic pattern of enhancement. The rate of activity increased rapidly, corresponding to increasing Mg$^{++}$ concentrations upto 20 mM, before levelling off. At concentrations above 22.5 mM, the rate again increased and finally levelled at about 40 mM Mg$^{++}$.

Figure 4 clearly shows that GS in *S. platensis* had two distinct binding sites for Mg$^{++}$ (denoted as I and II), having $K_m$ values of 4.8 mM and 8.8 mM, respectively. From the Hill plot, it was found that the binding of Mg$^{++}$ to the two sites was non-cooperative, as both I and II have 'n' values of unity (Figure 4, insets).
TABLE I. Effect of Mg$^{++}$ on reverse transferase activity

<table>
<thead>
<tr>
<th>Divalent cation (mM)</th>
<th>Specific activity $^b$</th>
<th>Crude extract</th>
<th>Purified enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn$^{++}$ (3)</td>
<td>0.746</td>
<td>20.66</td>
<td></td>
</tr>
<tr>
<td>Mg$^{++}$ (60)</td>
<td>0.110</td>
<td>3.66</td>
<td></td>
</tr>
<tr>
<td>Mn$^{++}$ (3) + Mg$^{++}$ (60)</td>
<td>1.04</td>
<td>31.00</td>
<td></td>
</tr>
</tbody>
</table>

b. Specific activity is expressed as U, mg Protein$^{-1}$. 
TABLE II. Effect of various nitrogen sources on Mg\(^{2+}\) enhancement of GS.

<table>
<thead>
<tr>
<th>Nitrogen Source(^a) (mM)</th>
<th>Percent enhancement(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate (10)</td>
<td>37.1</td>
</tr>
<tr>
<td>(30)</td>
<td>48.5</td>
</tr>
<tr>
<td>Ammonium (2)</td>
<td>44.8</td>
</tr>
<tr>
<td>(5)</td>
<td>41.8</td>
</tr>
<tr>
<td>Glutamate (10)</td>
<td>48.8</td>
</tr>
<tr>
<td>Serine (10)</td>
<td>40.2</td>
</tr>
<tr>
<td>Alanine (10)</td>
<td>43.6</td>
</tr>
</tbody>
</table>

\(^a\) Nitrogen source in growth medium.

\(^b\) Results are expressed as percent enhancement of enzyme activity caused by the addition of 60 mM Mg\(^{2+}\) to the reaction mixture.
TABLE III. Effect of ammonium shock on Mg\(^{++}\) enhancement of GS

<table>
<thead>
<tr>
<th>Ammonium(^a) shock (min.)</th>
<th>Mg(^{++}) (60 mM) in reaction mixture</th>
<th>Specific Activity (U.mg protein(^{-1}))</th>
<th>Percent enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>0.82</td>
<td>56.3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>+ (0)</td>
<td>+</td>
<td>1.23</td>
<td>54.8</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>+ (15)</td>
<td>+</td>
<td>1.29</td>
<td>64.4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.81</td>
<td>63.3</td>
</tr>
<tr>
<td>+ (30)</td>
<td>+</td>
<td>1.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.80</td>
<td>50.0</td>
</tr>
<tr>
<td>+ (45)</td>
<td>+</td>
<td>1.21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.80</td>
<td>54.20</td>
</tr>
<tr>
<td>+ (60)</td>
<td>+</td>
<td>1.24</td>
<td></td>
</tr>
</tbody>
</table>

\(a\). 200 ml of a normal nitrate grown culture (end log phase) was subjected to an ammonium shock of 30 mM. 30 ml aliquots were withdrawn at successive time intervals (in brackets) and harvested as described in Materials and Methods. Crude extract of nitrate grown cells, which did not undergo ammonium shock, served as control.
### TABLE IV. Effect of SVP treatment of ammonium shocked cells on Mg$^{++}$ enhancement

<table>
<thead>
<tr>
<th>Ammonium shock</th>
<th>SVP Treatment</th>
<th>Mg$^{++}$ (60 mM) in reaction mixture</th>
<th>Specific activity (U mg protein$^{-1}$)</th>
<th>Percent enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td></td>
<td>-</td>
<td>0.808</td>
<td>47.7</td>
</tr>
<tr>
<td>-</td>
<td></td>
<td>+</td>
<td>1.190</td>
<td></td>
</tr>
<tr>
<td>+ (0)</td>
<td>+</td>
<td></td>
<td>0.791</td>
<td>51.2</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td></td>
<td>0.791</td>
<td>46.6</td>
</tr>
<tr>
<td>+ (0)</td>
<td></td>
<td>+</td>
<td>1.158</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td>0.779</td>
<td>50.2</td>
</tr>
<tr>
<td>+ (30)</td>
<td></td>
<td></td>
<td>0.804</td>
<td>45.0</td>
</tr>
<tr>
<td>+ (30)</td>
<td></td>
<td></td>
<td>1.166</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td></td>
<td>0.779</td>
<td>48.6</td>
</tr>
<tr>
<td>+ (30)</td>
<td></td>
<td></td>
<td>0.808</td>
<td>48.0</td>
</tr>
</tbody>
</table>

---

a. 100 ml of nitrate grown cells were divided into two lots. One lot was harvested directly. The other lot was subjected to a 30 mM ammonium shock for a period of one hour and then harvested.

b. Crude extracts were subjected to SVP treatment. Extract containing 2 mg protein was incubated with 0.05 units of SVP at pH 8.0, at 37°C for 30 min. 50 ul samples were removed for the assay of GS activity. Controls which did not undergo SVP treatment were included.
TABLE V. Effect of Co\textsuperscript{++}, Cu\textsuperscript{++} and Ni\textsuperscript{++} on Mn\textsuperscript{++}-dependent reverse transferase activity

<table>
<thead>
<tr>
<th>Divalent cations in reaction mixture\textsuperscript{a}</th>
<th>Specific activity (U/mg protein\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mn\textsuperscript{++} (Control)</td>
<td>24.00</td>
</tr>
<tr>
<td>Cu\textsuperscript{++}</td>
<td>0.45</td>
</tr>
<tr>
<td>Mn\textsuperscript{++} + Cu\textsuperscript{++}</td>
<td>0.30</td>
</tr>
<tr>
<td>Co\textsuperscript{++}</td>
<td>1.25</td>
</tr>
<tr>
<td>Mn\textsuperscript{++} + Co\textsuperscript{++}</td>
<td>1.95</td>
</tr>
<tr>
<td>Ni\textsuperscript{++}</td>
<td>0.30</td>
</tr>
<tr>
<td>Mn\textsuperscript{++} + Ni\textsuperscript{++}</td>
<td>0.75</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Reaction mixtures were as described earlier except that Mg\textsuperscript{++} was replaced by 60 mM concentrations of Cu\textsuperscript{++}, Co\textsuperscript{++} or Ni\textsuperscript{++}, where indicated. Mn\textsuperscript{++} concentration was kept constant (3 mM). 44 fold purified enzyme was used in this experiment.
Figure 1. Changes in GS activity with varying Mn++ concentrations in the absence (○—○) and in the presence (●—●) of 60 mM Mg++. Details of the reaction mixture are given earlier. Inset gives the percent enhancement caused by 60 mM Mg++ with increasing concentration of Mn++(transferase assay).

Figure 2. Effect of varying pH on Mg++ enhancement of Mn++ transferase activity. Buffer system used over the pH range were MES (pH 5.5-6.5); Imidazole HCl (pH 6.5-7.5) Tris HCl (pH 7.5-8.5). The buffer concentration in the transferase assay was 50 mM. Activity in the absence (○—○) and presence (●—●) of 60 mM Mg++.

Figure 3. Plot of the rate of Mg++-dependent enhancement of GS activity. Reaction mixtures contained 3 mM Mn++ and varying concentrations of Mg++ (0-60 mM). Data given is an average of three independent experiments, each done in triplicates (transferase assay).
Figure 4. Eadie-Hofstee plot of data from Figure 3. Insets depict the Hill plot for I (○○) and II (●●) using the same data.
DISCUSSION

The presence of a covalent modification system is well known in gram negative bacteria (Shapiro and Stadtman, 1970b). In gram positive bacteria, however, this regulatory system does not appear to be operative (Wedler and Hoffmann, 1974a&b; Wedler et al, 1980). There is no evidence for adenylylation-deadenylylation regulation of GS in the filamentous nitrogen fixing cyanobacterium Anabaena, nor in the non-nitrogen fixing Anacystis nidulans (Rowell et al, 1977; Stacey et al, 1979; Emond et al, 1979).

Initial attempts to detect differences in the adenylylation states of GS in Klebsiella aerogenes and Rhodopseudomonas capsulata, as a function of the nitrogen sources, gave negative results. It was then shown by Bender et al, (1977), that the adenylylation state of K. aerogenes could change markedly during the harvesting of the cells. This could be prevented by pre-treatment of the cultures with the cationic detergent, CTAB. Similar results were found to hold true for R. capsulata (Johansson and Gest, 1977). Thus to prevent changes from occurring during harvesting, CTAB must be used. To ensure that no such changes occurred during the harvesting of S. platensis cultures, harvesting was done after treating the cells with 0.01% CTAB for 60 seconds.

An easy method used extensively as an estimate of the degree of adenylylation of GS utilizes the reverse transferase assay.
In the presence of 3 mM Mn\(^{2+}\) and 60 mM Mg\(^{2+}\), the assay is a measure of the activity of unadenyllylated enzyme. Without the addition of Mg\(^{2+}\), the assay is a measure of total GS activity, independent of the adenylylation state (Stadtman et al., 1970). Thus, the ratio of the activities, with and without Mg\(^{2+}\), provide an approximate measure of the degree of adenylylation of the enzyme. Using this technique in *S. platensis*, we observed a significant enhancement in GS activity, irrespective of the nitrogen source used for growth.

Nitrate has been reported to be the preferred inorganic nitrogen source for the growth of *Spirulina*. However, it was observed that the alga could also grow on ammonium, up to concentrations of 10 mM. The best growth was observed when ammonium was limiting (1-2 mM), growth on 10 mM ammonium was very poor. At concentrations higher than that, cell lysis occurred within a couple of days. Thus, when ammonium was used as a nitrogen source, it could support growth only up to 5 mM concentrations.

It is a well-documented fact that bacterial GS becomes highly adenylylated after shock treatment of nitrate grown cultures with high ammonium concentrations (Holzer et al., 1968). Therefore, normal nitrate grown *S. platensis* cells in culture were subjected to such a shock, using 30 mM ammonium over a period of one hour. However, this shock did not alter the response of GS to Mg\(^{2+}\).

The AMP moiety associated to GS in the adenylylated state could be cleaved by treating the enzyme with snake venom phosphodiesterase (SVP), resulting in active unadenyllylated GS. SVP treatment of crude
extracts from cells grown on nitrate and ammonium, and ammonium shocked cultures, however, had no effect on the Mg$^{++}$ enhancement of GS activity.

Reports of reproducible stimulation by Mg$^{++}$ have been made in the case of wild type Salmonella typhimurium extracts, grown on minimal medium (Kustu and McKeregram, 1975). Similar results were also reported for the wild type gram negative bacteria Klebsiella aerogenes (Prival et al, 1973). Cell free preparations of these bacteria (known to contain a covalent modification system) apparently had more 'unadenylylated' activity than 'total' activity, due to stimulation of the activity of the 'unadenylylated' wild type enzyme in the presence of Mg$^{++}$. This situation, however, was observed only under conditions which yielded highly unadenylylated GS. Such a stimulation was not observed in conditions that produced adenylylated GS. In S. platensis, we observed a Mg$^{++}$ induced enhancement of GS activity under all conditions, including those which would be expected to yield adenylylated GS.

All these results clearly indicate that the covalent modification control mechanism is absent in Spirulina platensis. They also show that this cyanobacterial GS is uniquely affected by Mg$^{++}$, under all the conditions examined.

In the absence of adenylylation/deadenylylation cascade control mechanism, GS from this cyanobacterium is regulated by a number of other factors viz., end production inhibition, feedback inhibition by some amino acids, nucleotides, metabolic intermediates and divalent cations. In conjunction with these observations, the stimulation caused by magnesium could have some significance. Results in Chapter II showed
that an increase in the free Mg$^{++}$ resulted in an increased sensitivity to feedback inhibition of the biosynthetic activity. However, increase in Mg$^{++}$, in the Mn$^{++}$-dependent transferase assay, resulted in substantial enhancement of transferase activity. It is possible that the binding of Mg$^{++}$ could also cause changes in the substrate binding patterns.

Glutamine has been found to inhibit biosynthetic activity effectively at non-physiological concentrations. It is possible that the binding of Mg$^{++}$ to the enzyme could increase the affinity of glutamine to the enzyme (alter its $K_m$ apparent) such that it could cause inhibition at much lower concentrations, making the enzyme more sensitive to end product inhibition by glutamine.

Increase in Mg$^{++}$ concentrations also reflects the energy charge of the cell. An increase in free Mg$^{++}$ occurs when there is a lowering of the energy charge implying increased relative levels of AMP, which in turn could also potentiate glutamine binding to the enzyme, resulting in the inhibition of GS.

In addition, we have a number of interesting observations on the enhancement pattern exhibited by Mg$^{++}$. The Mg$^{++}$ mediated enhancement was not constant. The greatest stimulatory effect was observed when Mn$^{++}$ was at a concentration that produced maximal enzyme activity.

Stimulation also occurred over a wide pH range, but dropped to zero at pH 8.5. Since this cyanobacterium is normally grown in alkaline pH range (8.0-9.0), this may have some significance in that under normal conditions this enhancement may not occur but under conditions of stress or under more acidic environments, free Mg$^{++}$ fluctuations could influence enzyme activity in more than one way.
The possibility that Mg\(^{++}\) has a regulatory influence on GS is supported by the fact that there is a biphasic pattern of enhancement. The Eadie-Hofstee plot and the Hill plot of the biphasic curve clearly indicate that the enzyme has two binding sites for Mg\(^{++}\), each with a distinct K\(_m\) value. The two sites show no cooperativity in the binding of Mg\(^{++}\). Lastly, the enhancement caused by Mg\(^{++}\) could not be reproduced by other divalent cations, ruling out the possibility that this is a general divalent cation effect.

The importance of Mg\(^{++}\) as a regulatory factor for enzyme activity has been emphasized by Blair (2), who proposed that fluctuations in free Mg\(^{++}\) concentrations are an expression of the adenylate charge and regulated energy utilization in the cell. It is clear that Mg\(^{++}\) has a profound effect on GS in _S. platensis_, but, at this stage, we are unable to offer a plausible mechanism for its possible role in GS regulation.