Glutamine Synthetase of Pea Leaves

DIVALENT CATION EFFECTS, SUBSTRATE SPECIFICITY, AND OTHER PROPERTIES

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ABSTRACT

Purified glutamine synthetase from pea seedlings was most active with Mg\(^{2+}\) as the metal activator, but Mn\(^{2+}\) and Co\(^{2+}\) were 45 to 60% and 30 to 45% as effective, respectively, when assayed at the optimal pH for each cation. The Mg\(^{2+}\) saturation curve was quite sigmoid, and evidence indicates that MgATP is the active ATP substance. Co\(^{2+}\) also gave a sigmoidal saturation curve, but when Mn\(^{2+}\) was varied only slightly sigmoidal kinetics were seen. Addition of Mn\(^{2+}\), Ca\(^{2+}\), or Zn\(^{2+}\) at low concentrations sharply inhibited the Mg\(^{2+}\)-dependent activity, partially by shifting the pH optimum. Addition of Co\(^{2+}\) did not inhibit Mg\(^{2+}\)-dependent activity. The nucleotide triphosphate specificity changed markedly when Co\(^{2+}\) or Mn\(^{2+}\) replaced Mg\(^{2+}\). Using the Mg\(^{2+}\)-dependent assay, the Michaelis constant (Km) for NH\(_3\) was about 1.9 x 10\(^{-4}\) M. The Km for L-glutamate was directly proportional to ATP concentration and ranged from 3.5 to 12.4 mM with the ATP levels tested. The Km for MgATP also varied with the L-glutamate concentration, ranging from 0.14 mM to 0.65 mM. Ethylenediaminetetraacetic acid activated the enzyme by up to 54%, while sulfhydryl reagents gave slight activation, occasionally up to 34%.

MATERIALS AND METHODS

The methods were the same as described in our earlier paper (16). However, in the present study an additional assay method was employed; namely, the transfer assay. In this assay the ability of the enzyme to catalyze the following reaction was measured:

\[
L-\text{Glutamine} + \text{NH}_3 + \text{ADP, M}^2+ \rightarrow \text{ATP, M}^2+\text{Pi, or AsO}_4^- \rightarrow \gamma-\text{glutamyl hydroxamate} + \text{NH}_4^+\]

\(\gamma\)-Glutamyl hydroxamate was determined colorimetrically by the procedure described previously (16). The components of the reaction mixture are given in Table VI.

In all cases, the enzyme used in the present studies was at least 75% pure and usually homogenous as determined by disc gel electrophoresis. The enzyme preparations were stored for periods of up to 3 months.

RESULTS

Except where noted, the results below were similar using either the modified Pi assay (16) or the glutamyl hydroxamate assay (16).

Metal Ion Specificity. The purified enzyme had an absolute requirement for a divalent cation, and Mg\(^{2+}\) gave the optimal activity. Mn\(^{2+}\) and Co\(^{2+}\) were 45 to 60% and 30 to 45% as effective, respectively, but with Co\(^{2+}\) and especially Mn\(^{2+}\), the ratio of concentration of divalent cation and ATP was critical; this ratio strongly influenced the pH optimum (16). Zinc and Fe\(^{2+}\) gave less than 5% of maximal activity, although no studies were made to determine condition (such as pH optimum) for optimal activity with these cations. Calcium gave only about 1% of \(V_{max}\) (pH 7.8), and acted as a potent inhibitor when present with Mg\(^{2+}\) or Mn\(^{2+}\).

Several combinations of divalent cations were tested. Mg\(^{2+}\) plus Mn\(^{2+}\). When Mg\(^{2+}\) was present at a concentration of 20 mM (ATP, 8 mM) or 50 mM, the addition of as little as 0.25 mM Mn\(^{2+}\) caused marked inhibition (Fig. 1), when assayed at pH 7.8. At pH 6.2, however, the addition of 0.5 mM Mn\(^{2+}\) stimulated the reaction by at least 100% (ATP, 8 mM, MgSO\(_4\), 20 mM), but only at lower levels (10 mM) of L-glutamate. With 30 mM glutamate, the addition of 0.25 to 1.0 mM Mn\(^{2+}\) caused some inhibition, but much less than that which occurred at pH 7.8 under the same conditions. Part of the inhibition caused by Mn\(^{2+}\) at pH 7.8 is due to a change in pH optimum, from 7.8 to 8.0 (Mg\(^{2+}\) alone) to below 5.8 (Mg\(^{2+}\) plus Mn\(^{2+}\)) (Fig. 2). This is similar to the optimum pH for Mn\(^{2+}\) alone (where \([\text{Mn}^{2+}] = [\text{ATP}]\) which is about 5.2 (16).

Where Mn\(^{2+}\) was kept constant (pH 6.2) the addition of Mg\(^{2+}\) had little effect if Mn\(^{2+}\) was not rate-limiting, but where Mn\(^{2+}\) was limiting (below one-third \(V_{max}\)) the addition of 5 to 7.8 under the same conditions.

Glutamine synthetase has been intensively investigated in *Escherichia coli* with respect to substrate specificity, metal ion specificity, reaction mechanism, and physical properties. The enzyme has been shown to have a number of rather unusual properties including pronounced effects of different divalent cations on kinetic and regulatory properties, and a high degree of nonspecificity with regard to L-glutamate analogues (8, 13, 19, 20, 26, 27). The *Bacillus subtilis* rat liver, and ovine glutamine synthetases have been similarly investigated (4–6, 14, 18, 21) although not as intensively.

With respect to higher plants, the pea seed enzyme has been purified and partially characterized (23, 25), as have the carrot (3) and rice (9) enzymes. An earlier paper (16) described the preparation of purified glutamine synthetase from pea leaves, which is perhaps distinct from the pea seed enzyme. In this paper, further information concerning its kinetic properties are reported.

1 This work was supported by a grant from the National Research Council of Canada.
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10 mM Mg$^{2+}$ stimulated the reaction severalfold. Probably the pH also influences these interactions.

**Mg$^{2+}$ plus Co$^{2+}$.** In the presence of saturating levels of Mg$^{2+}$ (pH 7.8) the addition of Co$^{2+}$ was much less inhibitory than Mn$^{2+}$, but resulted in a shift of pH optimum to 6.5 to 6.8 (Fig. 3), which is the optimal pH for Co$^{2+}$ alone (16).

**Mg$^{2+}$ plus Ca$^{2+}$ or Zn$^{2+}$.** At pH 7.8 (no EDTA, Mg$^{2+}$, 20 mM, ATP, 8 mM) the addition of 5 mM Ca$^{2+}$ caused 85% inhibition and shifted the pH optimum from 7.8 to between 6.9 and 7.1. Even at this pH, Ca$^{2+}$ still caused 70% inhibition. Zn$^{2+}$ (2 mM) caused 70% inhibition at pH 7.8 under analogous conditions. Where Ca$^{2+}$ was 10 mM (ATP, 6 mM, pH 6.7), the addition of 18 mM Mg$^{2+}$ increased activity 40%, and even this concentration of Mg$^{2+}$ was not saturating.

**Mn$^{2+}$ plus Co$^{2+}$.** Where Mn$^{2+}$ was limiting (Mn$^{2+}$, 1 mM, pH 6.2, ATP, 6 mM), the addition of Co$^{2+}$ caused over 50% stimulation, with the optimal Co$^{2+}$ level being 8 to 10 mM. Where cobalt was saturating the addition of Mn$^{2+}$ was not nearly so inhibitory as when Mn$^{2+}$ was added to Mg$^{2+}$ assay (Fig. 4), perhaps due to the lower pH optimum with Co$^{2+}$.

**Mn$^{2+}$ plus Ca$^{2+}$.** Where Mn$^{2+}$ was presented at 3 mM (ATP, 6 mM, pH 6.2), the addition of 3 mM Ca$^{2+}$ caused 60% inhibition, with a shift of pH optimum from 6.2 to below 5.6. However, at pH 5.2 where Mn$^{2+}$, 11 mM (ATP, 8 mM) the addition of 1 mM Ca$^{2+}$ caused only 4% inhibition.

**Concentration of Divalent Cations.** When Mg$^{2+}$ concentration was varied there was a definite sigmoidal response (Fig. 5), and as the ATP was increased the sigmoidal response remained but higher concentrations of Mg$^{2+}$ were needed to produce maximal activity.

Where the Mg$^{2+}$/ATP ratio is 1:1, such that almost all of the Mg$^{2+}$ should be complexed to ATP (24), the reaction is still stimulated 40 to 60% by increasing amounts of Mg$^{2+}$ (Fig. 5), and the stimulation by excess (over ATP) Mg$^{2+}$ was even more striking where d-glutamate was used.

It is difficult to assess the Mn$^{2+}$ concentration dependence, for as [Mn$^{2+}$] increases, the pH optimum decreases, although the shift is small as long as [Mn$^{2+}$] is equal to or less than 0.5 [ATP$^{+}$] (16). It can be seen (Fig. 6) that the Mn$^{2+}$ saturation curve is not sigmoidal as is the case with Mg$^{2+}$, and that even where [Mn$^{2+}$] is only one-third [ATP] there is near maximal activity. The decrease in activity when Mn$^{2+}$ is greater than the 4 mM is due to a shift in pH optimum (16). At pH 5.2,
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Fig. 3. Effect of Co\(^{2+}\) on the pH optimum of the Mg\(^{2+}\)-dependent activity. The reaction contained conditions as in Figure 2, except 10 mM CoCl\(_2\) replaces MnSO\(_4\).

Mn\(^{2+}\) can be increased to 5 to 6 mM in excess of ATP with no inhibition (16).

The same problems occur when varying Co\(^{2+}\) as with Mn\(^{2+}\), in that the pH optimum varies, although it is a much broader optimum than with Mn\(^{2+}\) (16). The Co\(^{2+}\) saturation curve is sigmoidal and is more similar to the Mg\(^{2+}\) curve than to the Mn\(^{2+}\) curve.

Nucleotide Triphosphate Specificity. With purified enzyme the nucleotide specificity varied greatly depending on the divalent cation used. With Mg\(^{2+}\), ATP was most effective, but with Mn\(^{2+}\), ITP and UTP were almost as effective; while with Co\(^{2+}\) the situation was intermediate (Table I). The Km values and possible influence of pH on specificity were not investigated.

The apparent Km values for ATP, determined under a variety of conditions, are given in Table II. It is difficult to measure the Km for ATP with Mn\(^{2+}\), for as already discussed, the pH optimum shifts strongly as the level of ATP varies above or below the Mn\(^{2+}\) level. However, by providing a constant 1 mM excess of Mn\(^{2+}\) at each level of ATP the pH opti-
the reaction with D-glutamate required a higher Mg\(^{2+}\) concentration than L-glutamate. It is not known if this is true for other analogues. The relative activity data above are approximate, for interacting variables such as pH, Mg\(^{2+}\), ATP, and NH\(_4\)OH concentrations were not extensively examined. Using Mn\(^{2+}\) (pH 6.2, 7 mM ATP, 3 mM ATP, 3 mM Mn\(^{2+}\), 8 mM NH\(_4\)OH) the relative activities with L-glutamate, α-methyl Dl-glutamate, and D-glutamate were 100%, 54%, and 28%, respectively (all at 40 mM concentration).

The apparent Km for L-glutamate varies with ATP concentration such that the values (pH 7.8, 20 mM Mg\(^{2+}\), 5 mM NH\(_4\)Cl) at 0.4 mM, 1.2 mM, and 8 mM ATP were 3.5 mM, 6.6 mM, and 12.4 mM, respectively (Table III).

Several glutamate analogues were ineffective. There was no activity where 20 to 30 mM acetyl-L-glutamate, carbamyl aspartate, or L-aspartate replaced L-glutamate (pH 7.8, Mg\(^{2+}\), assayed by Pi liberation). Several L-glutamate analogues inhibited the enzyme when tested in the presence of L-glutamate (Table IV). Methionine sulfoxide was much more effective inhibitor when Mn\(^{2+}\) was the divalent metal ion. The nature of the inhibition by methionine sulfoxide and methionine sulfoximine will be discussed later.

Amine Donor Specificity. With the pea leaf enzyme at all stages of purification, NH\(_4\)OH and NH\(_4\)Cl worked equally well with L-glutamate at pH 6.2 (Mn\(^{2+}\)) or pH 7.8 to 7.9 (Mg\(^{2+}\)). Varner (22), using the pea seed enzyme, noted that with D-glutamate, NH\(_4\)OH was much more effective than NH\(_4\)Cl, and the optimum NH\(_4\)OH concentration varied with pH.

The Km for NH\(_4\)Cl was difficult to determine, for it was so low that the assays employed lacked desirable sensitivity. By using the modified Fiske-SubbaRow assay system described previously (16), and reading at 333 nm, or by using the coupled spectrophotometric assay, the Km for NH\(_4\)Cl was found to be approximately 1.5 to 2.2 × 10\(^{-3}\) M at pH 7.9 (Mg\(^{2+}\)). Using Mn\(^{2+}\) in place of Mg\(^{2+}\), the Km for NH\(_4\)Cl was much higher, 0.8 to 0.9 mM, when ATP and glutamate were saturating. Other Activators and Inhibitors. EDTA and DTPA\(^{2-}\), at low concentrations, usually increased the activity of the enzyme by 30 to 60% when used with Mg\(^{2+}\), but the magnitude of the effect varied between preparations and perhaps with the age of the preparation (Table V). The optimal DTPA level was between 0.5 and 1 mM, while with EDTA, 4 to 5 mM gave maximum stimulation, which might reflect the greater binding constant of DTPA for divalent cations (1). The decline in reaction rate which occurred at higher levels of these chelators is probably due to removal of Mg\(^{2+}\).

Mercaptoethanol and other sulfhydryl compounds usually increased the activity of the enzyme, but the stimulation with mercaptoethanol at pH 7.8 (Mg\(^{2+}\)-dependent activity) varied from 0 to 34%. The stimulation by mercaptoethanol was greatest when EDTA or DTPA were present. GSH gave only 0 to 11% stimulation while cysteine caused 2 to 15% stimulation. The optimal amounts of cysteine and mercaptoethanol varied between 4 to 12 mM, and at higher concentrations they decreased activity. No stimulation by mercaptoethanol was observed where the Mn\(^{2+}\)-dependent activity (pH 5.2 or 6.2) was assayed.

Transfer Assay. Although this reaction was not studied in great detail, some general information was obtained. At pH 7.4 it was found that 0.2 mM ADP was about 90% saturating (Mg\(^{2+}\), 20 mM, K\(_2\)HPO\(_4\), 25 mM, NH\(_4\)OH, 12 mM, L-glutamine, 50 mM). The activity with Mg\(^{2+}\) versus Mn\(^{2+}\) and AsO\(_4\)\(^{3-}\) is shown in Table VI, where it is seen that a combination of Mn\(^{2+}\)

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8 Abbreviations: DTPA: diethylthiurammoniumpentaacetate acid; Bicine: N,N-Bis(2-hydroxyethyl)glycine; PIPES: pipazine-N,N'-bis(2-ethanesulfonic acid).
and AsO₄²⁻ gave the greatest activity. Using Mn²⁺ and AsO₄²⁻, the pH optimum was between 6.2 and 6.6 (MES-KOH buffer), while at pH 5.4 and 7.8 (Tricine-KOH buffer) the relative activities were 74% and 40%, respectively, of the pH 6.4 activity.

At pH 7.4 (5 mM Mn⁺⁺) the Km for AsO₄²⁻ was approximately 2 mM. With 20 mM Mg²⁺ the Km for AsO₄²⁻ was between 5 and 10 mM. It must be emphasized that Km values for PO₄³⁻, AsO₄²⁻, Mg²⁺, and Mn²⁺ are not too reliable, for significant precipitation of magnesium or manganese salts of phosphate and arsenate sometimes occurs, thus lowering the effective concentrations.

The Km for L-glutamine in this assay was not determined, but 50 mM was saturating. L-asparagine was not utilized by the enzyme. ATP, or other di- and triphosphate nucleotides were not tested in the transfer assay.

In all cases, the activity at optimal Mn²⁺ and AsO₄²⁻ levels at pH 6.2 to 6.6 was at least seven to eight times that for the biosynthetic assays (Mg²⁺, pH 7.8) and was even greater relative to the biosynthetic assay with Mn²⁺.

Table III. Apparent Km Values for L-Glutamate and Some Analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cation</th>
<th>Aminic Source</th>
<th>pH</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Glutamate</td>
<td>Mg²⁺ (20)</td>
<td>NH₄Cl (6)</td>
<td>7.8</td>
<td>11.4-13</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>Mg²⁺ (20)</td>
<td>NH₂OH (10)</td>
<td>7.8</td>
<td>9.3-9.9</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>Mn²⁺ (3-4)</td>
<td>NH₂OH (5)</td>
<td>6.2</td>
<td>3.8-5.8</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>Mn²⁺ (3.5-4)</td>
<td>NH₂OH (10)</td>
<td>6.2</td>
<td>4.4-5.6</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>Mn²⁺ (10)</td>
<td>NHCl (5)</td>
<td>5.2</td>
<td>8.7</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>Mn²⁺ (10)</td>
<td>NHOH (10)</td>
<td>5.2</td>
<td>11.0</td>
</tr>
<tr>
<td>d-Glutamate</td>
<td>Mg²⁺ (25)</td>
<td>NH₂OH (48)</td>
<td>7.8</td>
<td>10.0</td>
</tr>
<tr>
<td>d-Glutamate</td>
<td>Mg²⁺ (5)</td>
<td>NH₂OH (5)</td>
<td>7.4</td>
<td>15.6</td>
</tr>
<tr>
<td>d-Glutamate</td>
<td>Mn²⁺ (3)</td>
<td>NH₂OH (8)</td>
<td>6.2</td>
<td>8.6</td>
</tr>
<tr>
<td>α-Methyl DL-glutamate</td>
<td>Mn²⁺ (3)</td>
<td>NH₂OH (8)</td>
<td>6.2</td>
<td>13.5</td>
</tr>
<tr>
<td>α-Methyl DL-glutamate</td>
<td>Mg²⁺ (25)</td>
<td>NH₂OH (48)</td>
<td>7.8</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Table IV. Inhibition of Enzyme by Several L-Glutamate Analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cation</th>
<th>pH</th>
<th>Concentration of L-glutamate</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (L-glutamate)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>Carbamyl aspartate (6)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>50</td>
<td>102</td>
</tr>
<tr>
<td>Acetyl glutamate (6)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>50</td>
<td>102</td>
</tr>
<tr>
<td>L-Methionine S-sulfoxide (6)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>L-Methionine S-sulfonamide (6)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>50</td>
<td>4</td>
</tr>
<tr>
<td>Control L-glutamate</td>
<td>Mn²⁺ (3.5)</td>
<td>6.1</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Carbamyl aspartate (12)</td>
<td>Mn²⁺ (3.5)</td>
<td>6.1</td>
<td>10</td>
<td>101</td>
</tr>
<tr>
<td>Acetyl glutamate (12)</td>
<td>Mn²⁺ (3.5)</td>
<td>6.1</td>
<td>10</td>
<td>97</td>
</tr>
<tr>
<td>L-Methionine S-sulfonamid (12)</td>
<td>Mn²⁺ (3.5)</td>
<td>6.1</td>
<td>10</td>
<td>19</td>
</tr>
<tr>
<td>L-Methionine S-sulfonamide (8)</td>
<td>Mn²⁺ (3.5)</td>
<td>6.1</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>L-Aspartate (33)</td>
<td>Mn²⁺ (3.5)</td>
<td>6.2</td>
<td>12</td>
<td>73</td>
</tr>
</tbody>
</table>

**DISCUSSION**

One of the most unusual properties of the pea leaf glutamine synthetase is the effect of divalent cation species and their concentration on the substrate specificity and kinetic behavior of the enzyme. An earlier paper (16) reported the pronounced differences in pH optimum, varying from 5.2 to 8.3, depending on the divalent cations used and their concentrations. In the present paper it is seen that the divalent cation species also strongly influences the nucleotide triphosphate specificity, where Mn²⁺ results in low specificity. The Bacillus subtilis enzyme is also less specific for nucleotide triphosphate with Mn²⁺ than with Mg²⁺ (4).

Although Mg²⁺ results in greatest activity (with ATP), Co²⁺ gives good activity, and Mn²⁺ was capable of yielding 45 to 60% of maximal activity. At lower pH values, or where [Mg²⁺] is less than [ATP], Mn²⁺ or Co²⁺ actually give greater activity than Mg²⁺. It has also been found with most bacterial and animal glutamine synthetases that Mg²⁺, Co²⁺, and Mn²⁺ are the
Table V. Effect of Chelating Compounds on Glutamine Synthetase Activity

Assayed by glutamyl hydroxamate biosynthetic reaction when the components were ATP, 8 mM; L-glutamate, 60 mM; and NH₄OH, 10 mM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cation</th>
<th>pH</th>
<th>Relative Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA (0.5)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>133</td>
</tr>
<tr>
<td>DTPA (0.5)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>138</td>
</tr>
<tr>
<td>EDTA (4.0)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>154</td>
</tr>
<tr>
<td>DTPA (4.0)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>144</td>
</tr>
<tr>
<td>EDTA (12)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>98</td>
</tr>
<tr>
<td>DTPA (12)</td>
<td>Mg²⁺ (20)</td>
<td>7.8</td>
<td>77</td>
</tr>
<tr>
<td>DTPA (25)</td>
<td>Co²⁺ (10)</td>
<td>6.7</td>
<td>86</td>
</tr>
<tr>
<td>DTPA (25)</td>
<td>Mn²⁺ (3)</td>
<td>6.2</td>
<td>85</td>
</tr>
<tr>
<td>DTPA (25)</td>
<td>Mn²⁺ (3)</td>
<td>6.2</td>
<td>40</td>
</tr>
<tr>
<td>DTPA (5)</td>
<td>Mn²⁺ (3)</td>
<td>6.2</td>
<td>2</td>
</tr>
</tbody>
</table>

Table VI. Properties of the Transfer Assay

The reaction was run for 6 min at 35 C. The reaction mixture contains 0.1 mM Tricine-KOH buffer, pH 7.4, 50 mM L-glutamate, 0.5 mM ADP, 12 mM NH₄OH, and 1.5 µg of purified enzyme.

<table>
<thead>
<tr>
<th>Cation</th>
<th>Anion</th>
<th>Glutamyl Hydroximate Produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µM</td>
<td>µM</td>
</tr>
<tr>
<td>Mg²⁺ (20)</td>
<td>PO₄³⁻ (25)</td>
<td>0.32</td>
</tr>
<tr>
<td>Mg²⁺ (20)</td>
<td>AsO₄³⁻ (20)</td>
<td>0.80</td>
</tr>
<tr>
<td>Mn²⁺ (4)</td>
<td>PO₄³⁻ (25)</td>
<td>0.30</td>
</tr>
<tr>
<td>Mn²⁺ (4)</td>
<td>AsO₄³⁻ (20)</td>
<td>1.24</td>
</tr>
<tr>
<td>Mn²⁺ (8)</td>
<td>AsO₄³⁻ (20)</td>
<td>1.59</td>
</tr>
</tbody>
</table>

three most effective cations (7, 8, 14, 19, 26, 27). With the pea seed enzyme, where M⁺/ATP = 1, Varner (22) found that the relative activities with Mg²⁺, Co²⁺, and Mn²⁺ were 100%, 110%, and 21%, respectively. This contrasts with the data presented in this paper, where at pH 7.5 Co²⁺ is only 25% as effective as Mg²⁺. The carrot enzyme (pH 7.5) was also most active with Mg²⁺, followed by Co²⁺ (67%), Fe³⁺ (30%), and Mn²⁺ (25%) (3).

Calcium and Mn²⁺ have both been found to inhibit the Mg²⁺-dependent activity of the glutamine synthetase from most organisms (21). Part of the inhibition caused by Ca²⁺ and especially Mn²⁺ in the present study is due to the shift in pH optimum which results, but this is not the whole explanation. It is probable that Mn²⁺, Ca²⁺, and perhaps Zn²⁺ are competing with Mg²⁺ and have a much higher binding constant, but possibly these ions have binding sites distinct or partially distinct from the Mg²⁺ binding sites(s). In this respect, the E. coli enzyme apparently has three independent binding sites, where the high affinity Mn²⁺ site bound Mn²⁺ nearly 400-fold more readily than it bound Mg²⁺, while the medium affinity site bound both ions equally well (19). These same data suggested that Mg²⁺, Mn²⁺, and Co²⁺ all bind to the same high affinity site (19). The Mn²⁺ activation of the Mg²⁺-dependent activity at low pH (6.2) has not been reported in other systems, and may reflect an effect of pH on the relative binding constants for Mg²⁺ versus Mn²⁺, but it is also influenced by the glutamate concentration.

The sigmoid saturation curves observed for Mg²⁺ and Co²⁺ have not, to our knowledge, been seen with this enzyme from other plant sources (3, 22, 23, 25). This has been observed, however, with the unadenylated E. coli enzyme (19) and was even more pronounced with Bacillus subtilis (4). The sigmoid kinetics observed here probably mean that MgATP is the actual substrate of the enzyme, while the evidence points to the existence of a separate Mg²⁺ site or sites as well. That either Mg²⁺ or ATP can bind to the enzyme without the other is shown in the stability data reported earlier (16), and in unpublished data.

The inhibition observed here with the glutamate analogues, methionine sulfoxide and methionine sulfoximine, seems to be a general property of most glutamine synthetases (13, 21), including the carrot enzyme (3). Data have been presented which indicate that they inhibit by being phosphorylated and then binding tightly to the active site (12).

The stimulation by EDTA, DTPA, and sulphydryl compounds was observed only with Mg²⁺, which is another example of the effect of divalent cation source on the enzyme properties. Such stimulation by sulphydryl compounds was reported to occur in some preparations of pea seed enzyme, but not in others (25).

The pea leaf enzyme exhibits strong glutamyl transferase activity, as does the seed enzyme (23), and Mn²⁺ and AsO₄³⁻ were much more effective cofactors than Mg²⁺ or PO₄³⁻. Likewise, the apparent Km values reported here are similar to those reported for the pea seed enzyme (18, 22). The pea leaf enzyme had about the highest Km for L-glutamate (at saturating ATP) yet reported (up to 13 mM) compared to 3 to 6 mM for the pea seed enzyme (18, 22), 3.8 mM for the rice enzyme (9), and 3.7 mM for the carrot (3). The interesting difference between the pea seed and pea leaf enzyme is that the former has a Km for MgATP which is 18-fold higher than that for l-glutamate (22), while for the pea leaf enzyme they are similar. The apparent Km values for ATP reported here (saturating glutamate) are similar to those reported for carrot (3), rice (9), and E. coli (27).

The Km values for both glutamate and ATP are directly proportional to the concentration of the other, which indicates the probability of substrate competition between the two, either at catalytic sites or possibly at other (distinct) sites.

The Km for NH₄⁺ varies widely with the enzyme source, and is also known to vary widely with different l-glutamate analogs and pH, at least with the pea seed enzyme (22). The value obtained in the present study is similar to the two values obtained by two laboratories using pea seed enzyme, namely 1.3 × 10⁻³ M (18) and 5 × 10⁻⁴ M (22). These Km values are much less than those reported for the carrot enzyme (15) (Km NH₄⁺ ≥ 2.2 mM). The rice enzyme (3) had a Km for NH₄OH of 4 × 10⁻⁴ M, and the pumpkin enzyme 6.7 × 10⁻⁴ M (11). It is rather surprising to note the 20- to 100-fold difference in Km for NH₄⁺ (or NH₄OH) for the glutamine synthetase of different plant sources. Another point worth emphasizing is the high affinity of most plant glutamine synthetases for NH₄⁺ compared to the very low affinity for NH₄⁺ of glutamic dehydrogenase from various plant sources, where the Km varies from 0.01 to 0.1 M (2, 10). This fact, together with the generally severalfold greater activity of glutamine synthetase over glutamic dehydrogenase in the same plants points to the likelihood that glutamine synthetase is an especially effective sink for NH₄⁺ in higher plants, preventing toxic concentrations of NH₄⁺ from accumulating, particularly in the chloroplast, where this enzyme has been located (15).

Perhaps the most interesting and complex characteristic of the pea leaf glutamine synthetase which the present and previous (16) results illustrate is the very great influence of divalent cation species and concentration on the pH optima, substrate specificity (especially nucleotide triphosphates), apparent Km...
values (for NH₄⁺), and the general catalytic activity of the enzyme. The enzyme must be capable of existing as a variety of conformers in response to interactions between pH, divalent cation source, and the relationship of that cation to the ATP concentration, divalent cation combinations, and other substrate concentrations.

The modulation of activity by Mn²⁺ at any pH could possibly play an important regulatory role in vivo, leading to either an increase or decrease in activity, or a decrease in pH optimum, or both. In addition, Mn²⁺ has a pronounced effect on certain end product inhibition patterns which will be the subject of a later paper.

It would be interesting to determine whether the pea leaf enzyme contains bound Mn²⁺ as does the E. coli enzyme, where Mn²⁺ is believed to play a structural role in binding the subunits together (8, 26). Segal and Stadtman (19) found that with the E. coli enzyme there exists a distinct conformational state with each of the four cations (Mg²⁺, Co²⁺, Cd²⁺, and Ca²⁺), and the different cations caused different kinetic properties. The pea leaf enzyme conformation appears to be affected similarly by different cations, as suggested by kinetic data presented in this paper and by additional data to be the subject of another paper.

There are significant differences between the properties of the pea leaf and the pea seed enzymes. They are similar with respect to specific activity, Km for ATP and NH₄⁺, and pH optimum versus Mg²⁺ concentration. They are different with respect to the following properties: (a) apparent lack of sigmoidicity of Mg²⁺ or Co²⁺ saturation curves in pea seed enzyme; (b) the apparently greater stability of the pea seed enzyme; (c) the much higher Km for D-glutamate and the high ratio of Km D-glutamate/L-glutamate for the pea seed enzyme; (d) the fact that certain purification procedures which were highly effective with the pea seed enzyme were of little or no value in purification of the pea leaf enzyme; and (e) the pea seed enzyme appeared much more active with Co²⁺ than was the pea leaf enzyme.

At this point it cannot be stated whether these differences occur because the pea seed and leaf enzymes are actually different or simply reflect differences brought about by purification procedures, storage, or some unknown variables.

LITERATURE CITED

Corrections

Vol. 55: 251–257. 1975
III. The Action of Streptomycin on the Synthesis of Chlorophyll and Anthocyanin.
Pages 252 and 256, Figures 1 and 4 were reversed. The correct figures and legends follow.

![Graph 1](image1)

**Fig. 1.** Action of streptomycin on anthocyanin and Chl synthesis in cabbage seedlings. D: dark control; L: R + FR for 24 hr.

![Graph 2](image2)

**Fig. 4.** Action of ethanol on the synthesis of anthocyanins and Chl in cabbage seedlings. Light treatment: 24 hr, white (CWI); DC: dark control.

Page 257, references 21 and 22 refer to Annali di Botanica (Rome).

Vol. 54: 773–779. 1974
Page 775, Figure 5 legend should be corrected to read: X: 10 mM ATP.
CORRECTIONS

Vol. 54: 773-779. 1974
Page 778, Table VI legend, line 2, should be corrected to read: contains 0.1 M Tricine-KOH buffer, pH 7.4, 50 mM L-glutamine.

Vol. 54: 877-885. 1974
Fellows, Robert J. and Donald R. Geiger. Structural and Physiological Changes in Sugar Beet Leaves during Sink to Source Conversion.
Page 883, column 2, paragraph 2, lines 7 to 14, should be corrected to read: The plasmodesmata between sieve elements and companion cells of chemically fixed material (Fig. 4E) appeared to have a callose cylinder of about 290 nm in diameter with a dense interior core of about 200 nm, somewhat larger than those observed by Robards (29). Plasmodesmata between se and cc in freeze-substituted tissue (Fig. 4F) possessed little or no callose. They had an average outside diameter of about 270 nm with a clear center of about 200 nm.

Vol. 55. 1120-1122. 1975
Mizrahi, Yosef, Herbert C. Dostal, William B. McGlasson, and Joe H. Cherry. Transplantation Studies with Immature Fruit of Normal, and rin and nor Mutant Tomatoes.

Vol. 56. 207-212. 1975