Importance of Glutamate Synthase in Glutamate Synthesis by Soybean Cell Suspension Cultures

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ABSTRACT

The specific activities of glutamate synthase [EC 2.6.1.53, L-glutamine: a-ketoglutarate amino transferase (NADPH-oxidising)] and glutamine synthetase [EC 6.3.1.2, L-glutamate: ammonia ligase (ADP-forming)] extracted from soybean (Glycine max L.) cells grown in modified B5 medium were found to vary significantly in response to variations in the nitrogen content of the medium. The changes seen in specific activity levels could be correlated with similar patterns seen in the growth of the cells, in response to changes in the nitrogen content of the medium. By contrast, the specific activity of glutamate dehydrogenase [EC 1.4.1.2, L-glutamate: NAD+ oxidoreductase (deaminating)], was relatively low and invariant. Glutamate synthase was extracted from cells grown under optimal conditions, partially purified, and shown to have many properties in common with preparations of this enzyme extracted from other plant sources. Glutamate synthase was purified to homogeneity, using affinity chromatography on blue Sepharose.

Glutamate synthase was originally discovered in bacteria (16), but has since been shown to be present in algae (9), and a variety of higher plant tissues (12). A recent review article (12) has discussed evidence which suggests that the main pathway for ammonia utilization and glutamate formation in higher plant tissues is via the combined action of glutamine synthetase and glutamate synthase. Such discussions have been based on considerations of the relative K_M values for ammonia exhibited by glutamine synthetase and glutamate dehydrogenase; the response of plants to inhibitors of glutamine metabolism, and the results of pulse-chase experiments.

The experiments described here were designed to illustrate the relative importance of the three enzymes: glutamate synthase, glutamine synthetase, and glutamate dehydrogenase, in the synthesis of glutamate in soybean cells grown in suspension culture. Experiments were designed to detect any variations occurring in the relative specific activity levels of these three enzymes, when concentrations of nitrogenous constituents of the growth medium were altered. Some properties of a partially purified preparation of glutamate synthase were studied, and a method found for purifying this enzyme to homogeneity using affinity chromatography.

MATERIALS AND METHODS

CHEMICALS

DON was a gift from D. Blair of the Cancer Medical Research Institute, University of Saskatchewan. Azaserine was purchased from Calbiochem. Purified L-glutamine (99% grade) was obtained from All Tech Associates, Arlington Heights, Ill. Asparagine was obtained from the Sigma Chemical Co. Various agaroagarsehexane NAD+ or NADP+ derivatives were obtained from P-L Biochemicals Inc., Milwaukee, Wis. Blue Sepharose CL6B and Sephadex G-50 (medium grade) were purchased from Pharmacia, Canada Ltd., Montreal, Quebec. All other chemicals used were of the highest purity obtainable from commercial sources.

PURIFICATION OF CHEMICALS

L-Asparagine was separated from contaminating aspartic acid by chromatography on Dowex AG 1-X10 (acetate form) ion exchange resin.

Polyclur AT obtained from Chemical Developments of Canada Ltd., Point Clair, Quebec, was purified according to the method of Loomis and Battaille (10).

THIN LAYER CHROMATOGRAPHY

Mixed cellulose and silica gel thin layers were prepared using a mixture of 7.2 g of MN-Silica Gel G (Canadian Laboratory Supplies, Winnipeg, Manitoba), and 18 g of Sigma Cell type 38 microcrystalline cellulose (Sigma Chemical Co.) homogenized in 144 ml of distilled H_2O. This mixture was spread 0.25 mm thick over glass plates (20 × 20 cm), which were then oven-dried at 80 C for approximately 24 hr. Solvent systems used for the separation of amino acids were: phenol-water (80:20, v/v), L-butanoy-acetic acid-water (80:20:20, v/v/v), and methanol-pyridine-water (20:1:5, v/v/v). Amino acids on thin layer plates were detected by spraying with 0.03% (w/v) ninhydrin in 3% acetic acid in L-butanol (v/v). After spraying, the plates were dried in an oven at 115 C.

Mixtures of organic acids were separated using a solvent system prepared from ether-formic acid-water (7:2:1, v/v/v). After drying thoroughly enough to remove all traces of formic acid, organic acid spots were detected using a spray reagent prepared from 0.04% (w/v) bromoresol green in ethyl alcohol, with the addition of 0.1 N NaOH until a blue color was just produced. After spraying lightly with this reagent, plates were developed by holding over a dish of dilute ammonia.

AMINO ACID ANALYSIS

The analysis of amino acids in some enzyme reaction mixtures was performed by M. Farmer of the Department of Animal and Poultry Science, University of Saskatchewan, using a Beckman automated amino acid analyzer. Norleucine was used as an internal standard.

POLYACRYLAMIDE GEL ELECTROPHORESIS

Analytical polyacrylamide gel electrophoresis was carried out using the system described by Davis (4). Gels were stained using...
a solution of 1.25 g of Coomassie brilliant blue G-250 dye dissolved in 227 ml methyl alcohol, to which was added 46 ml acetic acid, and water to 500 ml. Staining was carried out for 1 hr and gels were then rinsed and destained using 7.5% acetic acid in 5% methyl alcohol (v/v).

PLANT CELL CULTURES AND THEIR MAINTENANCE

Suspension cultures of soybean (Glycine max L. var. Mandarin) were grown routinely in B5 medium as described previously (6, 7). Cells were also maintained as callus cultures on B5 medium solidified with 0.65% agar. Dry weights were determined as previously described (15).

COMPOSITION OF ALTERED B5 MEDIUM USED FOR GROWTH EXPERIMENTS

To determine the response of the plant cells to growth in medium containing different nitrogen sources, cell cultures were grown for 96 hr in B5 medium to which the following changes had been made.

Culture Group 1. Ammonium sulfate was supplied in concentrations ranging from 0.2 mM to 100 mM, with potassium nitrate initially at 25 mM.

Culture Group 2. L-Glutamine was supplied in concentrations from 0.2 to 100 mM, with potassium nitrate initially at 25 mM.

Culture Group 3. Potassium nitrate was supplied in the 2 mM to 100 mM concentration range, with ammonium sulfate initially at 1 mM.

KCl was added to these cultures to maintain the initial K level of the medium at 25 mM. With each sample, from each of the above groups, determinations were made of dry weight and wet weight. In addition, the specific activities of the enzymes glutamate synthase, glutamate dehydrogenase, and glutamine synthetase were determined upon crude cell extracts, whose preparation is described in the next section.

ENZYMES EXTRACTION AND PURIFICATION

Preparation of Crude Extracts. The buffers used throughout this section were as follows. Extraction buffer consisted of 200 mM Tricine, 2 mM EDTA, 0.1% (v/v) mercaptoethanol (pH 7.5). Equilibration buffer consisted of 25 mM Tricine, 1 mM EDTA, 0.1% (v/v) mercaptoethanol (pH 7.5). The amounts and quantities of cells and buffer hereafter described were those used for the preparation of quantities of crude extract suitable for the purification of glutamate synthase.

Cells were collected by suction filtration through Miracloth (Calbiochem) and then washed with B5 medium containing 0.1% (v/v) mercaptoethanol. Fifteen-g aliquots of cells were taken for homogenization using a Braun MSK homogenizer (Canadian Laboratory Supplies, Winnipeg, Manitoba). Each aliquot of cells was homogenized for 90 sec with 45 g of glass beads (1.0 mm) and 15 ml extraction buffer in a 75-ml stopped glass container cooled to 4°C. The homogenate obtained was filtered through Miracloth and the filtrate centrifuged at 27,000g for 15 min at 4°C; the supernatant obtained is termed crude extract.

Further Purification of Glutamate Synthase. We found that glutamate synthase activity could be concentrated in a 40 to 60% ammonium sulfate fraction, with no significant loss of activity. The 40 to 60% fraction was dissolved in 24 ml of equilibration buffer, and desalted by passage through a column of Sephadex G-50, measuring 1.7 x 32 cm, and eluted with equilibration buffer. The various affinity media previously described were tested for their ability to bind glutamate synthase by stirring aliquots of Sephadex G-50-treated enzyme with affinity medium in a centrifuge tube for 15 min at 4°C. At the end of this time each mixture was centrifuged at 12,000g for 15 min, and the supernatant used for enzyme assays and protein determination.

ENZYME ASSAY METHODS

Glutamate synthase was assayed at 30°C by measuring the decrease in A at 340 nm following the oxidation of NADH or NADPH. Routine assay mixtures for glutamate synthase were constituted as follows: Tricine, 25 mM; L-glutamine, 10 mM; α-ketoglutarate, 1 mM; mercaptoethanol, 0.1% (v/v); and between 2.5 and 75 units of enzyme activity in a total reaction volume of 2.5 ml (pH 7.5). The reaction was initiated by the addition of enzyme, and the rate of O.D. change was linear for at least 3 min. In all assays except those using highly purified enzyme, some oxidation of NADH and NADPH was found to occur independently of the glutamate synthase reaction. This was assumed to be due to the presence of a reduced pyridine nucleotide oxidase. Consequently, the glutamate synthase activity in crude and partially purified enzyme extracts was determined as the difference in the NADH or NADPH oxidation rate with and without glutamine.

GLUTAMINE SYNTHETASE

Glutamine synthetase was measured according to the method of Kanamori and Matsumoto (8) with slight modifications as follows. The standard assay systems consisted of the following: Tricine, 25 mM; EDTA, 1 mM; mercaptoethanol, 0.1% (v/v); ATP, 0.05 mM; hydroxylamine, 0.1 mM; cysteine, 0.1 mM; magnesium sulfate, 0.1 mM; L-glutamate, 0.5 mM; and 28 to 40 units of enzyme in a total volume of 3 ml. The reaction was initiated by addition of L-glutamate which was replaced in the blank by 0.5 ml of water. After incubation for 15 min at 30°C, the γ-glutamyl hydroxamate formed was determined by adding 1.0 ml of a ferric chloride reagent, prepared from equal volumes of 10% FeCl3-6 H2O in 0.2 N HCl, 24% trichloroacetic acid, and 50% HCl.

GLUTAMATE DEHYDROGENASE

Glutamate dehydrogenase activity was measured according to the method of Dougall (5).

DEFINITION OF ENZYME UNIT

One unit of enzyme activity is that amount of enzyme that will catalyze the transformation of 1 μmol of substrate/min at 30°C.

RESULTS

GROWTH CHARACTERISTICS OF CELLS IN DIFFERENT MEDIA

The optimal yield in terms of dry weight, packed cell volume, or wet weight of cells grown in standard B5 medium was found after 96 hr of growth. The levels of specific activity of glutamine synthetase, glutamate synthase, or glutamate dehydrogenase in the cell cultures were determined at various stages of growth (Fig. 1). Levels of glutamate dehydrogenase stay low and more or less constant, while the specific activities of glutamate dehydrogenase and glutamine synthetase rise to very high values by 96 hr of growth.

The growth pattern of cells on B5 medium that had been modified in various ways was also studied. The medium was modified by either altering the level of nitrate present while keeping ammonium ion levels constant, or altering the concentration of ammonium ion while keeping nitrate constant, or including various concentrations of L-glutamine in the medium in place of ammonium, but keeping the nitrate levels constant. When ammonium sulfate levels were varied, between 0.2 and 100 mM (Fig. 2) then an optimum growth rate was seen at a 1-mM concentration of ammonium sulfate, equivalent to a 2-mM concentration of ammonium ion. When glutamine levels were varied over the same range (Fig. 2), an optimum growth rate was seen with 4 mM glutamine. The use of ammonium as a source of reduced nitrogen
Cells were glutamine described specific activity obtained from this cultures. These figures, Gamborg growth medium between 2 and a gave (A A), (U--), the result is in (v--), synthetase, and glutamate dehydrogenase Plant

FIG. 1. Changes in the specific activities of glutamine synthetase ( )--( ), glutamate synthase with NADPH ( )--( ), and glutamate dehydrogenase with NADH ( )--( ), with NADPH ( )--( ), during different stages in the growth of a soybean cell cultures.

FIG. 2. Effect of varying (NH₄)₂SO₄ ( ), glutamine ( ), or NO₃ ( ) in cell growth medium on the growth rate of soybean cells. Cells were grown for 96 hr and then sampled.

The levels of specific activity of glutamate synthase, glutamine synthetetase, and glutamate dehydrogenase obtained from cells grown under the differing regimes of nitrate, and ammonium or glutamine described above, are displayed in Figures 3 to 5. From these figures, a number of important points are readily apparent. Glutamate dehydrogenase is conspicuous by the low levels of specific activity obtained from this enzyme, and by the absence of any significant fluctuations in these levels in response to fluctuations in the level of nitrogen-containing constituents in the medium. By contrast, the levels of glutamine synthetetase and glutamate synthase can be seen to fluctuate between quite wide limits in response to changes in the levels of the nitrogen-containing constituents of the medium. In particular, it can be seen (Figs. 3 and 4) that markedly increased levels of these two enzymes are obtained when using concentrations of ammonium and nitrate which produced optimal growth of the cells. When glutamine was the source of reduced nitrogen for the cells, the specific activity of glutamine synthetetase obtained from cells grown in the concentration of glutamine optimal for growth (4 mM) was approximately 60% of that seen at lower concentrations of glutamine (0.5 mM). By contrast glutamate synthase levels may be seen to rise to a value that is almost maximal at 4 mM glutamine (Fig. 5).

PURIFICATION OF GLUTAMATE SYNTHASE

Various conventional methods of enzyme purification including isoelectric precipitation, and heat treatment at 40 to 60 °C for varying periods of time, were utilized in an endeavor to increase the specific activity of glutamate synthase in crude cell extracts. Of the methods tried, precipitation between 40 and 60% saturation with ammonium sulfate was found to be useful for concentrating enzyme activity, without also giving a drastic loss of this activity. The method used by Beevers and Storey (2) for partial purification of glutamate synthase from pea cotyledons was not successful in our hands when used with soybean cells; the final 100,000g supernatant had copious quantities of reduced pyridine nucleotide oxidase activity still present.

The enzyme preparation produced by fractionation of crude cell extracts with 40 to 60% ammonium sulfate was termed partially purified, and was used for a number of purposes including further attempts at purification using affinity chromatography. Of the various affinity media tested, by far the best results were obtained with blue Sepharose. Results of preliminary experiments carried out to determine the binding efficiency of different Sepharose derivatives, showed that 60 to 70% of the total units in the partially purified enzyme preparation were bound to blue Sepharose. Further work showed that it was possible to purify the enzyme to homogeneity, using a column of blue Sepharose of 56 ml total volume (1.34 × 38.2 cm), equilibrated with equilibration buffer. A volume of 9 ml of the partially purified enzyme (1.34
units) was mixed with 1 ml of a 5 mM solution of L-glutamate, and then applied to the column, all operations taking place at 4°C. The column was now washed with equilibration buffer until the A at 280 nm of the column effluent approached that of the buffer. The enzyme was released from the column by applying 20 ml of equilibration buffer that was 50 mM in L-glutamate and 50 mM in α-ketoglutarate, also 0.2 M in NaCl (termed pulse salt mixture). This was followed by 200 ml of equilibration buffer to elute the released enzyme from the column. The peak fractions were either singled out, or pooled together to make distinct samples according to their A at 280 nm (Fig. 6 and Table I). Before assay these samples were passed through Sephadex G-50. Using this method, fractions which had significant glutamate synthase activity were found to give one band on polyacrylamide gel electrophoresis, and to be purified by 430-fold compared with crude enzymes extracts.

### PROPERTIES OF PARTIALLY PURIFIED AND PURIFIED ENZYME

**Substrate Specificity for Amino Group Donor.** The partially purified enzyme displayed maximal activity with glutamine as an amino group donor. Although almost as much activity was seen with commercial asparagine as with glutamine, when this asparagine was purified to remove aspartate, only about 2% of the original activity was retained. When glutamate synthase that had been purified by affinity chromatography was incubated with purified asparagine, no activity whatsoever was seen. Thus, it appears that asparagine cannot act as an amino group donor in the glutamate synthase-catalyzed reaction.

**Substrate Specificity for the Coenzyme Involved in Reduction.** Three compounds were tried as reducing coenzymes: NADH, NADPH, and reduced ferredoxin. Both NADH and NADPH could be utilized as reducing agents; highest enzyme activities were always seen with NADH rather than NADPH. The inability

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**FIG. 3.** Variation in specific activities of glutamate synthase ( ), glutamate dehydrogenase ( ), and glutamine synthetase ( ) accompanying the variation of (NH₄)₂SO₄ concentration in cell growth medium. NADH was used as the cofactor for glutamate synthase and dehydrogenase.

**FIG. 4.** Variation in specific activities of glutamate synthase ( ), glutamate dehydrogenase ( ), and glutamine synthetase ( ) accompanying the variation of NO₃⁻ concentration in cell growth medium. NADH was used as the cofactor for glutamate synthase and dehydrogenase.
GLUTAMATE SYNTHESIS BY SOYBEAN CELLS

FIG. 5. Variation in specific activities of glutamate synthase (●), glutamate dehydrogenase (○), and glutamine synthetase (■) accompanying the variation of glutamine concentration in cell growth medium. NADH was used as the cofactor for glutamate synthase and dehydrogenase.

FIG. 6. Elution profile of glutamate synthase from blue-Sepharose CL-6B. Volume of each individual fraction collected was 7.0 ml; fractions were examined for O.D. at 280 nm (▲), and glutamate synthase activity (●).

Table 1. Purification scheme for glutamate synthase with Blue Sepharose CL-6B.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Units/ml x 10^4</th>
<th>Total Protein (mg/ml)</th>
<th>Units/mg protein x 10^3</th>
<th>Yield %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>60</td>
<td>35.5</td>
<td>2130.0</td>
<td>6.6</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>40-60% (NH₄)₂SO₄</td>
<td>24</td>
<td>148.4</td>
<td>3681.6</td>
<td>17.7</td>
<td>167</td>
<td>3</td>
</tr>
<tr>
<td>Fraction eluted from Blue Sepharose CL-6B affinity column:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>14</td>
<td>6.2</td>
<td>43.4</td>
<td>0.16</td>
<td>38.8</td>
<td>2</td>
</tr>
<tr>
<td>23</td>
<td>14</td>
<td>5.0</td>
<td>35.0</td>
<td>0.08</td>
<td>22.5</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>14</td>
<td>4.0</td>
<td>101.5</td>
<td>0.01</td>
<td>1450.0</td>
<td>5</td>
</tr>
<tr>
<td>29 &amp; 30</td>
<td>14</td>
<td>14.2</td>
<td>108.0</td>
<td>0.005</td>
<td>2840.0</td>
<td>9.4</td>
</tr>
<tr>
<td>31, 32 &amp; 33</td>
<td>21</td>
<td>14.2</td>
<td>298.2</td>
<td>0.005</td>
<td>2840.0</td>
<td>14.1</td>
</tr>
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</table>
of ferredoxin to act as a reducing agent was determined by subjecting reaction mixtures containing this compound to TLC; examination of the chromatograms showed that no glutamate was produced.

**Substrate Specificity for the Amino Group Acceptor.** Both \( \alpha \)-ketoglutarate and oxaloacetate were examined as possible amino group acceptors. In both the spectrophotometric assay and in the production of glutamate seen on thin layer chromatograms, \( \alpha \)-ketoglutarate showed good activity as an amino group acceptor. Oxaloacetate showed no activity at all as an amino group acceptor, when used in conjunction with NADH. When NADPH was the coenzyme, very large amounts of enzyme activity appeared to be present when activity was measured spectrophotometrically. Such reaction mixtures did not show any formation of glutamate when they were examined chromatographically. When oxaloacetate was incubated with partially purified enzyme in the absence of any amino group donor, a rapid oxidation of NADPH (but not NADH) still occurred. The most probable explanation of this reaction is the presence of an NADP(H)-dependent malate dehydrogenase in the partially purified extracts, since glutamate formation could not be detected chromatographically.

**Optimal Substrate Levels and Michaelis Constants of Partially Purified Enzyme.** The concentrations of glutamine, \( \alpha \)-ketoglutarate, and reduced pyridine nucleotide that produce optimal enzyme activity were determined by keeping the concentration of two of these constant, while varying that of the third. The optimal concentrations determined in this manner were: NAD and NADPH, 0.3 mM; \( \alpha \)-ketoglutarate, 1 mM; and glutamine, 10 mM. The Michaelis constant for each substrate was determined using the method of Lineweaver and Burk, and using optimal rather than saturating levels of two substrates, while varying the level of the third substrate. This procedure was chosen since marked substrate inhibition was seen at levels above optimum with either of the pyridine nucleotides, and with \( \alpha \)-ketoglutarate. The \( K_M \) values obtained are shown in Table II.

**Reaction Stoichiometry.** When reaction mixtures containing purified enzyme, optimal amounts of \( \alpha \)-ketoglutarate, glutamine, and NADPH were examined using the amino acid analyzer, it was found that 1 molecule of glutamate and 1 molecule of \( \alpha \)-ketoglutarate yielded exactly 2 molecules of glutamic acid.

**pH Studies.** The effect of pH on reaction velocity was determined using samples of equilibration buffer prepared to cover a pH range from 3 to 11. Cofactors and substrates were dissolved in the buffer of appropriate pH at each stage. Aliquots of partially purified enzyme were added to initiate the reaction, and after allowing the reaction to proceed for 3 min, the pH was immediately measured using a Fisher expanded scale pH meter and microprobe electrode (Fisher Scientific Co., Edmonton, Alberta). The results obtained with NADH and NADPH are recorded in Figure 7.

**Enzyme Inhibition Studies.** Aliquots of partially purified enzyme containing 17.7 \( \times \) 10\(^{-3}\) to 74.2 \( \times \) 10\(^{-3}\) units of enzyme activity were incubated with 0.15 ml of inhibitor at 30 C for 15 min. Aliquots of 0.5 ml (0.4 ml enzyme + 0.1 ml inhibitor) were taken and added to the reaction mixture sitting in the cuvette chamber at 30 C, and reaction velocity then recorded (Table III).

**DISCUSSION**

When soybean cells were grown on various modified B5 media differing in the concentration, and type of the nitrogen sources available for growth, optimal growth of cells in each medium was confined to a relatively small concentration range of nitrate, ammonium, or glutamine. When the concentrations of the normal nitrogen-containing constituents of B5 medium (nitrate and ammonium) were varied, the specific activities of glutamine synthetase and glutamate synthase varied in a manner that resembled the growth pattern of the cells themselves (Figs. 2 to 5). When cell growth was optimal, high yields of enzymes would be obtained; when cell growth was poor, low yields of enzymes were obtained. By contrast to the results obtained with glutamine synthetase and glutamate synthase, the specific activity levels of glutamate dehydrogenase remained consistently low, and did not fluctuate significantly in response to growth conditions imposed on the cells.

These results plus those obtained from cells grown on regular B5 medium (Fig. 1) appear to support suggestions made by other authors (12) that the primary pathway for glutamate production from ammonia in plants is via glutamine synthetase and glutamate synthase. This idea gains additional support from the results obtained when glutamine was supplied as a source of reduced
nitrogen in place of ammonia. Levels of glutamine synthetase appeared to fluctuate in such a way that they would ensure an optimal level of glutamine in the cells, and subsequently an optimal synthesis of glutamate. As glutamine levels in the medium rose toward 4 mM (where optimal growth of cells is obtained), the level of glutamine synthetase dropped, suggesting repression of glutamine synthetase synthesis by these concentrations of glutamine (Fig. 6). The specific activity of glutamate synthase, however, reached a level that was essentially maximal at 4 mM glutamine. It appears that both enzymes respond in a characteristic manner to the increase in glutamine concentration of the medium from 0.2 to 4 mM. While glutamine synthetase appears to be repressed, glutamate synthase by contrast does not appear to be repressed. It is important to note the relative specific activity levels of glutamine synthetase and glutamate synthase, and to see that at all times glutamine synthetase levels are approximately 1,000 times greater than those of glutamate synthase. Provided the cells are supplied with sufficient nitrogen, then glutamine should never become rate-limiting for the glutamate synthase-catalyzed reaction.

The results discussed above also offer at least a partial explanation of the well known fact (1, 6, 7, 13, 14) that soybean cells require an optimal (2 mM) concentration of ammonium ion for proper growth. Since both glutamate synthase and glutamine synthetase activities display quite sharp and discrete optima at this concentration of ammonium ion (Fig. 3), such a growth response could be expected.

Kinetic studies carried out on partially purified glutamate synthase indicated that the Kₘ values for all of the substrates are quite small (Table II), and similar in magnitude to those obtained by other authors using enzyme preparations from plant tissues (3, 12). The lowest Kₘ value in this table is that for NADH which is 9.0 x 10⁻⁴ M, together with the higher enzymic activities seen with NAD and the higher Kₘ value for NADPH (5.7 x 10⁻⁵ M); this strongly suggests that NADH rather than NADPH is the physiologically active pyridine nucleotide for glutamate synthase from soybean cells. This result is similar to those obtained by other workers studying glutamate synthase from plant sources (2, 11). The pH activity curves (Fig. 7) also appear to indicate that NADH-dependent activity is expressed over a wider pH range than is NADPH-dependent activity. The soybean tissue culture cell enzyme differs from the pea root enzyme investigated by Miflin and Lea (11) in its complete inability to use ferredoxin. The exact significance of the use of ferredoxin by an enzyme from roots is not clear. Miflin and Lea (11) pointed out that the involvement of coupled diaphragm systems in their assays could not be ruled out.

Substrate specificity studies carried out with homogeneous enzyme preparations have shown that α-ketoglutarate and glutamine are the only amino group acceptor and donor molecules, respectively, utilized by the enzyme from soybean cell cultures. The results obtained by incubating partially purified soybean glutamate synthase with a variety of glutamine analogs (Table III) are consistent with this enzyme being able to utilize the amide group of glutamate as a source of an amino group for the formation of glutamate. Table III shows that almost complete inhibition of enzyme activity is obtained by incubation with DON and azaserine, while a significant degree of inhibition (23%) is seen with methionine sulfoximine. The apparent activity obtained with impure asparagine was not affected by the same concentration of DON that gave 95% inhibition of the glutamine-dependent reaction.

Affinity chromatography using blue Sepharose appears to offer a method of obtaining a significant purification of relatively crude glutamate synthase preparations. Since only one band staining with Coomassie blue was obtained on electrophoresis of the purified enzyme in polyacrylamide gel, it is probable that the preparation was in fact completely homogeneous. In this case, affinity chromatography offers a simpler method for the production of a homogeneous enzyme preparation than others previously published (3).

LITERATURE CITED
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