The synthesis of glutamine has been shown to occur in extracts of pigeon liver (1, 2) and of sheep brain (3, 4) by the following reaction.\(^1\)

\[
\text{Glutamic acid} + \text{ammonia} + \text{ATP} = \text{glutamine} + \text{phosphate} + \text{ADP}
\]

The enzyme system catalyzing this has been termed "glutamine synthetase" and for convenience this name will be used here. Ammonia in this reaction can be replaced by hydrazine or hydroxylamine, all three bases reacting at the same rate (4). All organic bases and amino acids tested have been found to be inactive. With hydroxylamine, a hydroxamic acid is produced, estimation of which provides a convenient colorimetric test for enzyme activity (5).

More recently, the work of Schou et al. (6) and of Stumpf et al. (7–9) has revealed another enzymic reaction leading to the formation of glutamylhydroxamic acid. The enzyme, which is known as glutamotransferase, catalyzes the exchange of the amide group of glutamine for either isotopic ammonia or hydroxylamine by the following reaction.

\[
\text{RCONH}_2 + \text{NH}_2\text{OH} = \text{RCONHOH} + \text{NH}_3 \quad (\text{RCONH}_2 = \text{glutamine})
\]

The reaction resembles that of glutamine synthesis in that amino acids and organic bases are unable to replace hydroxylamine as glutamyl acceptor. Transferase activity has been demonstrated in extracts of seedlings (7–9) and of pigeon liver and all mammalian tissues studied (6). With both types of tissues, phosphate or arsenate, Mn\(^{++}\) and trace amounts of either ATP or ADP were reported to be necessary for glutamotransferase activity. The adenine nucleotide acts catalytically in the transferase activity, in contrast to the glutamine-synthesizing reaction in which hydroxamic acid formation is paralleled by ATP breakdown. Waelsch et al. (10, 11) have in addition demonstrated in bacterial extracts both glutamo-
and aspartotransferase activities, forming the corresponding hydroxamic acids from glutamine and asparagine respectively. The bacterial transferases, however, appear to be of a different type from the glutamotransferase of plant and animal tissues in that no cofactors whatsoever are required for activity. No other aspartotransferase has yet been reported.

With both glutamine synthetase and glutamotransferase it has so far proved impossible to demonstrate any intermediate in the over-all reaction. Omission of any component of the glutamine-synthesizing system appears to result in complete inactivity. Thus, no phosphate liberation from ATP occurs if the enzyme is incubated with ATP + glutamate or with ATP + ammonia. Attempts to demonstrate the accumulation of an "active" glutamyl radical such as glutamyl phosphate have likewise failed (2, 4). In addition Speck (2) found that amidophosphate could not replace ATP + ammonia in glutamine synthesis. Stumpf et al. (7-9) have likewise been unable to detect an intermediate in the glutamotransferase reaction. It appeared desirable to obtain large amounts of the enzyme in concentrated solution as a preliminary to the exploration of the mechanism by which ATP breakdown is coupled with the condensation of glutamic acid and ammonia. This seemed necessary because of the possibility that the "active" intermediate in glutamine synthesis might not be readily dissociable from the enzyme-protein. In such a case, the initial reaction would presumably proceed only to the extent of the molarity of the enzyme present, or alternatively, would occur at a very slow rate. In either case large amounts of enzyme would be necessary to demonstrate such a reaction. The present work describes a method for the large scale preparation of highly purified synthetase and glutamotransferase from pea seeds. This preparation was undertaken to investigate whether one, or more than one, enzyme-protein is involved in glutamine synthesis.

As is described below, no separation of the glutamine synthetase and glutamotransferase activities was obtained throughout the purification procedure. Several experiments with the concentrated enzyme are also reported.

**Materials and Methods**

*Protein* was determined turbidimetrically; a 2 ml. sample was mixed with 3 ml. of 5 per cent trichloroacetic acid and after 30 seconds the absorption at 540 μm measured in the Klett-Summerson photometer. Values were taken from a standard curve made with crystalline bovine serum albumin.

*Measurement of Enzyme.* (a) *Glutamine Synthetase*—Incubations were carried out in open tubes for 20 minutes at 30°. The system contained 0.5 ml. of 0.8 M tris(hydroxymethyl)aminomethane buffer, pH 7.8, 0.5
ml. of 0.5 M Na glutamate, 0.5 ml. of 0.05 M ATP, 0.1 ml. of M MgSO₄, 0.1 ml. of M cysteine, 0.1 ml. of M NH₂OH at pH 7.5, and enzyme + water to final volume of 2.25 ml. The amount of enzyme was adjusted so that not more than 3 μM of hydroxamic acid were produced, under which conditions the value was proportional to the amount of enzyme added. After incubation the tubes were treated as described previously (4) and the hydroxamic acid measured (5).

(b) Glutamotransferase—Incubations were carried out as described above. Each tube contained 0.25 ml. of 0.01 M phosphate buffer, pH 6.5, 0.1 ml. of M MgSO₄, 0.1 ml. of M cysteine, 0.1 ml. of 0.01 M ATP, 1.0 ml. of 0.1 M L-glutamine, 0.1 ml. of M NH₂OH at pH 6.5, and enzyme + water to final volume of 2.25 ml. After incubation the hydroxamic acid was measured as for the synthetase.

Unit of Enzymes—For both activities an arbitrary unit of enzyme activity was defined as the production, under the standard test conditions, of 1.15 μM of glutamylhydroxamic acid (equal to a Klett-Summerson photometer reading of 100). Values were taken from a standard curve made with synthetic glutamylhydroxamic acid.¹

Phosphate Determination—Phosphate determinations were made by the Fiske and Subbarow method (12); the modifications introduced by Pett (13) were used for phosphate determinations in the presence of arsenate.

Chromatography of Adenine Nucleotides—Ascending chromatograms were made with Whatman No. 3 paper. The solvents used were (a) ethyl alcohol and 0.1 M sodium acetate-HCl buffer, pH 4.5, in equal volumes; (b) 5 per cent KH₂PO₄-isooamyl alcohol solvent (14). The nucleotides were detected by observation in ultraviolet light.

ATP was obtained as the disodium salt from the Pabst Brewing Company and ADP from the Sigma Chemical Company, as the barium salt. This was converted to the sodium salt by dissolving in HCl and precipitating the barium with sodium sulfate. The solutions were neutralized with NaOH.

Protamine sulfate and yeast nucleic acid were obtained from the Nutritional Biochemicals Corporation. The nucleic acid solutions were adjusted to pH 5.5 with KOH. Commercial samples of L-glutamic acid were used.

Hydrosylamine solutions were prepared from recrystallized hydrosylamine hydrochloride and neutralized to the required pH with NaOH.

A stock solution of cysteine hydrochloride was made and the samples neutralized with NaOH immediately before use.

Phosphate buffers were mixtures of K₂HPO₄ and KH₂PO₄. Imidazole

¹ I wish to express sincerely my thanks to Dr. H. Waelsch for a sample of synthetic glutamylhydroxamic acid.
buffers were prepared by adjusting the pH of solutions of the base with HCl.

Results

Purification of Enzyme

A typical preparation is described below.

Stage 1. Extraction—Dry green pea seeds (dwarf Blue Bantam variety) were purchased locally and pulverized to a fine powder in a power-driven hammer mill. 18 kilos of the powder were stirred for 30 minutes with 144 liters of cold 0.1 M NaHCO₃. Following this, 3.7 liters (0.05 volume) of 2 M MgSO₄ were stirred in and the precipitate was allowed to settle at 0° overnight. The supernatant fluid was poured off as cleanly as possible and the remaining suspension centrifuged on a centrifugal separator. The two supernatant fluids were combined. To avoid the centrifuging of large volumes, the suspension remaining after decantation can be rejected with a loss of only about 25 per cent of the enzyme. Volume of extract, 109 liters; protein, 26 mg. per ml.; synthetase, 2.3 units per ml.; specific activity (units per mg. of protein), 0.1; total units, 270,700; transferase, 2.0 units per ml.

The treatment with MgSO₄ removes gummy material which otherwise prevents satisfactory fractionation of the enzyme.

Stage 2. Fractionation with Ammonium Sulfate—The extract was adjusted to pH 6.5 by the addition of 2 M KH₂PO₄ and 300 gm. of solid ammonium sulfate were added per liter of extract. The precipitate was allowed to settle overnight at 0° and the supernatant fluid poured off and discarded. The precipitate was collected from the remaining suspension by centrifuging and resuspended in about 6 liters of cold distilled water. The thick suspension was brought to pH 7.2 with K₂HPO₄, put into cellophane tubes, and dialyzed against two changes of 40 liters of cold distilled water for about 36 hours. A small sample of the cloudy dialysate was centrifuged for enzyme assay; the main bulk was carried on to the next stage without centrifuging. Volume of extract, 8.7 liters; protein, 47 mg. per ml.; synthetase, 11.4 units per ml.; specific activity, 0.23; total units, 99,500; transferase, 11.0 units per ml.

Stage 3. Treatment with Protamine—The cloudy dialyzed extract was treated with a solution of 2 per cent protamine sulfate until a small sample, after centrifuging, gave no further precipitate on addition of a drop of protamine solution. About 2 liters were required. The bulky inactive precipitate was centrifuged and the supernatant fluid retained. Volume of extract, 10 liters; protein, 23 mg. per ml.; synthetase, 12.1 units per ml.; specific activity, 0.53; total units, 121,000; transferase, 9.1 units per ml.
The protamine treatment apparently removes an inhibitor from the extract, for an increase in total activity is usually obtained by this procedure.

Stage 4. Nucleic Acid Precipitation—The enzyme is completely precipitated around pH 5 by small amounts of yeast nucleic acid. 1 liter aliquots of the extract were treated at 0° with 10 ml. of M acetic acid (to pH 5.1), followed by 60 ml. of 2 per cent potassium nucleate solution (pH 5.5). The precipitate was centrifuged at 0° and suspended in a minimal volume of cold distilled water and neutralized to pH 7.3 of M K$_2$HPO$_4$, extracted for 15 minutes, and the suspension centrifuged. The precipitate was washed with cold dilute K$_2$HPO$_4$ and, after centrifuging, the two milky supernatant fluids were combined. Trial fractionation is performed for each batch of enzyme to determine the amount of nucleic acid required. Volume of extract, 400 ml.; protein, 40 mg. per ml.; synthetase, 227 units per ml.; specific activity, 5.7; total units, 90,800; transferase, 187 units per ml.

Stage 5. Second Ammonium Sulfate Fractionation—400 ml. of the enzyme solution were mixed with 16 ml. of M phosphate buffer, pH 7.4, followed by 288 ml. of saturated ammonium sulfate solution. The enzyme and ammonium sulfate solutions were previously cooled to 0°. After standing for 15 minutes, the bulky inactive precipitate was removed by centrifugation and a further 240 ml. of the saturated ammonium sulfate solution were added to the supernatant solution. After 20 minutes the precipitate was centrifuged and redissolved in cold water, with the addition of a few drops of M K$_2$HPO$_4$ to pH 7.3. Volume of extract, 64 ml.; protein, 18 mg. per ml.; synthetase, 820 units per ml.; specific activity, 50; total units, 52,000; transferase, 700 units per ml.

Stage 6. Dialysis—The solution was dialyzed with stirring against three changes of 4 liters of cold distilled water. The white precipitate was removed by centrifugation and the supernatant fluid was collected. Volume of extract, 75 ml.; protein, 6.1 mg. per ml.; synthetase, 590 units per ml.; specific activity, 97; total units, 44,300; transferase, 453 units per ml.

Stage 7. Second Nucleic Acid Precipitation—70 ml. of the enzyme solution were treated with 6.7 ml. of 1 per cent nucleic acid solution, followed by 1.3 ml. of 0.2 M acetic acid. The precipitate was collected and redissolved in dilute phosphate buffer, pH 7.3. As in the previous nucleic acid fractionation, a trial fractionation is carried out for each batch of enzyme by treating a small sample of enzyme with nucleic acid and adding graded amounts of acetic acid. At each stage the precipitate is collected by centrifugation and assayed for enzyme content. Volume of extract, 20 ml.; protein, 8 mg. per ml.; synthetase, 1760 units per ml.; specific activity, 220; total units, 35,200. The transferase activity at this stage was not determined. After storage at $-10^\circ$ for about 5 weeks, however, the ac-
tivity of the enzyme had decreased to approximately 50 per cent of the initial value. The solution at that stage contained 920 units of synthetase per ml. and 628 units of transferase.

The method of purification described here has been repeated a number of times on batches of about 150 to 200 liters of crude enzyme solutions without failure. A purification between 1000- and 2000-fold is consistently obtained. The purified enzyme contains more than a single protein component. Electrophoretic studies on the enzyme solution at pH 8.3 in Veronal buffer showed the presence of one main peak and two small subsidiary ones. In the ultracentrifuge at pH 7.3 in phosphate buffer, two approximately equal peaks were present.

Table I presents a summary of the purification procedure with respect to both the synthetase and transferase activities. It can be seen that

<table>
<thead>
<tr>
<th>Table I</th>
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<td>Summary of Purification Procedure</td>
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<table>
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<th>Stage No.</th>
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<th>Ratio, synthetase transferase</th>
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<td>30</td>
<td>1.0</td>
</tr>
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<td>3. Protamine</td>
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<td>37</td>
<td>1.3</td>
</tr>
<tr>
<td>4. 1st nucleic acid precipitation</td>
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<tr>
<td>5. 2nd ammonium sulfate precipitation</td>
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<td>16</td>
<td>1.3</td>
</tr>
<tr>
<td>6. Dialysis</td>
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<td>13</td>
<td>1.3</td>
</tr>
<tr>
<td>7. 2nd nucleic acid precipitation</td>
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<td>11</td>
<td>1.4</td>
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throughout the purification procedure the ratio of the activities of glutamine synthetase and glutamotransferase, under the standard test conditions, remained almost constant, indicating that no separation of the two activities existed. Furthermore it is observed in trial fractionations of various kinds that, whenever tested, the glutamine synthetase and glutamotransferase activities were present in the same fractions. In no case has one activity been observed in a preparation without the other being present.

The close association of the two activities, glutamine synthetase and glutamotransferase, raised the question whether these two activities are both the action of the glutamine-synthesizing system or whether they represent two unrelated enzymes with similar physical properties. A series of comparisons of the two activities was carried out.

Properties of Glutamine Synthetase and Glutamotransferase

Heat Stability—A sample of enzyme was heated to 45° and samples were removed at intervals for determination of activities. As shown in Table
II, the difference in rates of inactivation was very small and no satisfactory separation of the two activities was obtained.

Effect of pH—Fig. 1 shows the effect of pH on the synthetase and transferase activities. The former has an optimum at 7.5 and the latter at 6.75.

Effect of Metal Ions—Figs. 2 and 3 show the activation of glutamine synthetase and glutamotransferase by Mg$^{++}$ and Mn$^{++}$. In agreement with the findings of Stumpf et al. (8) and of Schou et al. (6), the enzyme is completely activated by 0.001 M Mn$^{++}$. The specificity towards Mn$^{++}$, however, is not complete, for Mg$^{++}$ at higher concentrations activates up to 60 per cent of the maximal activity with Mn$^{++}$. Suboptimal amounts of Mn$^{++}$ inhibit in the presence of Mg$^{++}$. With glutamine synthetase activity, the action of the two metals is reversed, the maximal activation given by Mn$^{++}$ being only 32 per cent of that with 0.04 M Mg$^{++}$.

### Table II

<table>
<thead>
<tr>
<th>Heated at 45°</th>
<th>Synthetase</th>
<th>Transferase</th>
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</tr>
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</tr>
<tr>
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</table>

**Table II**

Heat Inactivation of Glutamine Synthetase and Glutamotransferase

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** The effect of pH on the rate of glutamine synthetase and glutamotransferase. Imidazole buffer was used in all experiments. ⬤, synthetase; ○, transferase.
Inhibitors—Fluoride is a powerful inhibitor of both reactions, but glutamine synthetase activity is more sensitive than that of glutamotransferase. As is shown in Fig. 4, 0.001 M NaF inhibits the transferase 40 per cent and the synthetase 95 per cent. The pea enzyme resembles that from bacteria in being sensitive to the triphenylmethane dye, crystal violet, by contrast to the enzyme from sheep brain which is not affected (4, 15). At a concentration of 1:1000 the dye completely inhibits both the synthetase and transferase activities.
Effect of —SH Reagents—Cysteine at a concentration of 0.05 M has no effect on the glutamotransferase activity, but increases the activity of glutamine synthetase by 70 per cent. 0.001 M p-chloromercuribenzoate completely inhibits both reactions.

Experiments on Mechanism of Glutamine Synthesis

Incubations were carried out for 2 hours at 30° in the presence of 590 units of the purified synthetase enzyme to see whether release of inorganic phosphate from ATP could be detected from reactions other than the over-all glutamine-synthesizing reaction.

With the high concentration of enzyme a slight adenosinetriphosphate activity was observed. This release of phosphate was stimulated by the addition of 0.025 M potassium arsenate (11.9 μM of phosphate produced as compared with 3.8 μM in the absence of arsenate). This apparent “arsenolysis” of ATP was less than 0.2 per cent of the rate of glutamine synthesis. Addition of ammonia in the absence of glutamate did not cause a significant increase in ATP breakdown in either the presence or absence of arsenate. Glutamate usually did produce an increased liberation of phosphate, but, since this was reduced by taking care to exclude ammonia from the incubation mixture, the effect is probably due to contamination of the system with traces of ammonia and consequent synthesis of glutamine.

As described above, addition of ammonia in the absence of glutamine to a system containing large amounts of enzyme does not cause an increase
in ATP breakdown. The same is true of the other glutamyl acceptor, hydrazine. However, as shown in Fig. 5, hydroxylamine in relatively high concentration markedly stimulates the release of inorganic phosphate from ATP. No formation of a hydroxamic acid corresponding to the increased phosphate production could be detected. Chromatography of the incubation mixture after about 50 per cent of the total ATP present had broken down showed only two spots, corresponding in position to ATP.

**Fig. 5.** Effect of hydroxylamine on phosphate liberation from ATP by the purified glutamine synthetase enzyme. Incubations were carried out at 30° in tubes filled with N₂. The complete system contained 0.2 ml. of 0.5 M imidazole buffer, 0.05 ml. of 0.1 M MgSO₄, 0.2 ml. of 0.1 M NH₄OH, 0.2 ml. of 0.01 M ATP, 0.05 ml. of 0.1 M cysteine, and 0.3 ml. of purified enzyme (590 units of synthetase per ml.). After incubation, the solutions were deproteinized and phosphate estimated. ●, with NH₄OH; ○, no NH₄OH.

**Fig. 6.** Effect of enzyme concentration on the hydroxylamine-dependent liberation of phosphate from ATP by glutamine synthetase. Experimental conditions as described for Fig. 4. ●, with NH₄OH; ○, no NH₄OH.
and ADP. When ADP was present instead of ATP, the hydroxylamine effect on phosphate liberation did not occur. Fig. 6 shows the dependence on enzyme concentration of the hydroxylamine-dependent release of phosphate. The rate of the reaction is only about 0.5 per cent of the rate of glutamine synthesis.

Test for Coenzyme—Previous work has indicated that there is no coenzyme removable by simple dialysis which is involved in glutamine synthesis (2, 4). To test for firmly bound coenzyme, a sample of the purified enzyme was partially inactivated by drastic procedures such as heating and precipitation with ammonium sulfate at pH 3.0. After dialysis the activity of the enzyme so treated was not increased by addition of coenzyme A (CoA) or boiled pigeon liver extract. No CoA was found in a sample of enzyme (about 970 units of synthetase) which was boiled for 5 minutes and assayed by the pigeon liver acetylation system (16).

DISCUSSION

There is as yet insufficient evidence on which to propose a mechanism for the synthesis of glutamine. The results of purification, however, suggest that only a single protein component is involved in the reaction. This would imply that the enzyme catalyzes at least two reactions: (a) the “activation” of one of the components of glutamine (glutamate or ammonia) by a reaction with ATP and (b) the formation of glutamine from the intermediate and the second component of glutamine. The existence of the phenomenon of an enzyme catalyzing two distinct reactions has been clearly shown in the elegant studies of Harting and Velick (17) and of Racker and Krimsky (18) on the mechanism of triose phosphate dehydrogenation.

The failure to detect a reaction between ATP and either glutamate or ammonia suggests that the intermediate is firmly bound to the enzyme surface. This perhaps might be expected, if, in fact, a single enzyme carries out the entire synthesis.

With respect to the question of glutamine synthetase and glutamotransferase, the difference in properties of the two reactions, such as the effects of fluoride, cysteine, and metal activators, while supporting the concept of two distinct enzymes, cannot be regarded as conclusive (a) because the effects might be related to the difference of substrates present and (b) because of the possibility that the transferase reaction could conceivably represent a reversible reaction involving only the second step of glutamine synthesis. It is therefore not possible at this stage to decide whether the close association of the two activities is to be interpreted as a single enzyme catalyzing both reactions or as two distinct enzymes with similar physical properties.
SUMMARY

1. A method for isolating glutamine synthetase and glutamotransferase with a purification of 1000- to 2000-fold is described.

2. The relative activities, under standard assay conditions, of synthetase and transferase remained almost constant throughout the fractionation procedures.

3. The properties of the two activities are compared. The synthetase is maximally activated by Mg\(^{++}\) and to a smaller extent by Mn\(^{++}\); the reverse is true of the transferase. Differences in cysteine activation and fluoride inhibition are also obtained.

4. A number of experiments with concentrated enzyme are described.

5. The mechanism of glutamine synthesis is briefly discussed.

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W. H. Elliott

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