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Studies on the Mechanism of Glutamine Synthesis; Isolation and Properties of the Enzyme from Sheep Brain*

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Studies on the mechanism of the enzymatic synthesis of glutamine from glutamate, ammonia, and ATP have provided direct evidence for an enzyme-bound activated form of glutamic acid, the formation of which is associated with cleavage of ATP to ADP and phosphate. The present work was undertaken to develop a procedure for the isolation of relatively large amounts of highly purified enzyme for use in studies on the mechanism of the reaction. This report describes a procedure for the isolation of the enzyme from sheep brain, and gives some catalytic and physical properties of the enzyme. The purified enzyme, which is free of ammonia, inorganic phosphate, ATPase, and adenylate kinase, is at least 1000 times more active than the original brain homogenate, and it is essentially homogeneous on electrophoresis and ultracentrifugation. Sedimentation coefficients of between 14.5 and 15.1 *s*, and values of 430,000 and 497,000 for the molecular weight, were obtained. The enzyme is protected from thermal denaturation at 60° by ATP plus Mg^{++} (and to a lesser extent by ADP plus Mg^{++}). The concentration of ATP required for protection is of the same order of magnitude as the apparent K_m value for ATP in the catalytic reaction. The apparent K_m values for ammonia, hydroxylamine, L-glutamate, and D-glutamate were determined. Present observations on the optical specificity of the enzyme are consistent with the hypothesis, expressed earlier, that the mechanism of glutamine synthesis involves at least two steps, the second of which is much more optically specific than the first.

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Recent studies in this laboratory on the mechanism of the enzymatic synthesis of glutamine have provided direct evidence for the formation in the absence of ammonia of an activated form of glu-

tamic acid which is bound to the enzyme (Krishnaswamy *et al.*, 1960a, b; Meister *et al.*, 1961). The formation of the activated glutamate-enzyme complex is associated with cleavage of ATP to ADP and phosphate; both ADP and phosphate remain attached to the enzyme, and may be liberated by procedures that denature the protein. From this work it seems evident that experiments with relatively large quantities of the enzyme will be required to elucidate the mechanism further. These studies have also demonstrated the importance of using highly purified enzyme preparations that are virtually free of ammonia, inorganic phosphate, and ATPase.

The present communication describes a procedure for the isolation of the enzyme in highly purified form from sheep brain. The procedure makes use of the interesing fact that the enzyme is protected against thermal denaturation by ATP plus magnesium ions. The final preparation is free of ammonia and inorganic phosphate, and exhibits no detectable adenylate kinase or ATPase activity. It exhibits a specific activity which is at least a thousand-fold greater than that of the initial brain homogenate, and it is essentially homogeneous in the ultracentrifuge and on electrophoresis. Certain catalytic and physical properties of the enzyme are also given in this report.

Experimental

Materials.—ATP, the other nucleotides, and alumina Cγ gel were obtained from the Sigma Chemical Company. L-Glutamic acid was purchased from Schwarz BioResearch, Inc., and D-glutamic acid was obtained by the action of *Clostridium welchii* L-glutamic decarboxylase (Meister *et al.*, 1951) on DL-glutamic acid (Camien *et al.*, 1950). 2-Mercaptoethanol was an Eastman Kodak Company product. Ammonium sulfate was recrystallized from 0.001 M ethylenediaminetetraacetic acid. Calcium phosphate gel was prepared by the method of Singer and Kearney (1950).

Determination of Enzyme Activity.—Enzymatic activity was determined in a reaction mixture consisting of 50 μmoles of imidazole–HCl buffer (pH 7.2), 25 μmoles of 2-mercaptoethanol, 50 μmoles of magnesium chloride, 50 μmoles of L-glutamate, 100 μmoles of hydroxylamine hydrochloride (adjusted to pH 7.2 with NaOH), 10 μmoles of ATP, and enzyme, in a final volume of 1.0 ml. All of the reagents were added at 0°, and immediately after addition of ATP the reaction vessels were placed at 37°. After 15 minutes, the reaction was stopped by addition of 1.5 ml of a solution containing 0.37 M ferric chloride, 0.67 N hydrochloric acid, and 0.20 M trichloroacetic acid. After removal of the precipitated protein by centrifugation, the colors were compared with those given by authentic γ-glutamylhydroxamate in a Weston Colorimeter equipped with a 535 mμ filter. Under these conditions the assay was linear with time and enzyme concentration over the range 0.2 to 1.2 μmole. Controls in which enzyme and ATP were separately omitted were employed. When ammonium chloride was used in place of hydroxylamine, the reaction was followed by determination of the inorganic phosphate formed (Fiske and SubbaRow, 1925). Protein was determined by the procedure of Lowry *et al.* (1951), with crystalline bovine serum albumin used as the standard. A unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μmole of γ-glutamylhydroxamate under the conditions given above. Specific activity is expressed in terms of units per mg of protein.

Isolation of the Enzyme

Step 1. Preparation of Acetone-Dried Sheep Brain.—Fresh sheep brain was trimmed of gross connective tissue and homogenized in a large Waring blendor at 5° for 30 seconds at high speed. The homogenate was mixed with acetone previously cooled to −15° (2 liters per kg of brain). The suspension was ground vigorously in a large mortar for about 5 minutes or until the preparation was free of large lumps. The mixture was then poured into a large (32-cm diameter) Buchner funnel, and the residue was washed three times on the funnel with cooled acetone (total of 3 liters per kg of brain). The material was sucked dry and the powder was crumbled and allowed to dry in the air at room temperature for several hours. The dried powder was stored at −15°. Approximately 400 g of powder was obtained from 2.4 kg of fresh sheep brain.

Step 2. Extraction of Acetone powder.—Four hundred g of acetone powder was extracted, 200 g at a time, with a total of 4 liters of 0.005 M 2-mercaptoethanol containing 0.15 M KCl. The mixture was stirred mechanically at 26° for 10 minutes and then centrifuged at 4° at 13,000 g for 15 minutes. The supernatant solution was filtered at 4° through a thin layer of cotton and used immediately in the next step. (Subsequent steps were carried out at 4° unless otherwise stated.)

Step 3. Precipitation of the Enzyme at pH 4.2—The supernatant solution obtained in the previous step was cooled to 0° and divided into equal portions of approximately 1500 ml; each portion was adjusted to pH 4.2 by careful addition with mechanical stirring of 1.0 M acetic acid. Approximately 120 ml of acetic acid was added over a period of 20 minutes to each batch of 1500 ml. The precipitate which formed at pH 4.2 was immediately centrifuged at 13,000 × *g* for 15 minutes; the supernatant solution was discarded. The precipitates were combined and washed successively with 1 liter and 0.5 liter of 0.005 M 2-mercaptoethanol.

Step 4. Extraction at pH 6.8.—The washed precipitate was suspended in 200 ml of 0.005 M 2-mercaptoethanol containing 0.15 M potassium chloride, and the pH was adjusted to 6.8 by cautious addition of N NaOH at 0° (about 5 ml of alkali was required). The mixture was stirred mechanically for 30 minutes at 0° and then centrifuged at 13,000 × *g* for 30 minutes. The precipitate was re-extracted in the same manner, and the supernatant solutions were combined.

Step 5. Differential Heat Inactivation.—The following solutions were added to the supernatant solution obtained in the preceding step: 45 ml of 0.5 M imidazole-HCl buffer (pH 7.2)

containing 0.5 M $MgCl_2$, and 20 ml of an aqueous solution of 2.72 g of $ATP \cdot 3H_2O$ adjusted to pH 7 with sodium hydroxide. (The final concentrations of imidazole, ATP, and MgCl were 0.05, 0.01, and 0.05 M respectively.) The solution was placed in an 800-ml beaker immersed in a water bath maintained at 80°, and was stirred mechanically until the temperature inside the beaker reached 58° (approximately 4 minutes). This temperature was maintained for 6 minutes, during which time the solution became quite milky. The solution was then cooled rapidly to 4° and centrifuged at 13,000 $\times$ *g* for 15 minutes.

STEP 6. FRACTIONATION BY ADJUSTMENT OF PH.—The supernatant solution obtained in the preceding step was adjusted to pH 4.8 by cautious addition of 1.0 M acetic acid (about 20 ml was required) at 0°. The precipitate which formed was removed by centrifugation at 13,000 $\times$ *g* for 15 minutes, and discarded. The pH of the supernatant solution was adjusted to 4.2 by cautious addition of 1.0 M acetic acid (about 50 ml was required). The mixture was stirred mechanically for 30 minutes and then centrifuged at 15,000 $\times$ *g* for 30 minutes. The precipitate was washed with 200 ml of 0.005 M 2-mercaptoethanol. The supernatant solution obtained in this step was essentially devoid of enzymatic activity.

STEP 7. ADSORPTION ON CALCIUM PHOSPHATE GEL.—The precipitate obtained in the preceding step was dissolved in 5 to 10 ml of 0.005 M 2-mercaptoethanol. During this process, the pH was carefully adjusted to 7.0 by addition of 0.1 N sodium hydroxide; 0.005 M 2-mercaptoethanol was added to bring the final volume to 20 ml. This solution was dialyzed at 4° for 18 hours against 6 liters of 0.005 M 2-mercaptoethanol containing 0.001 M ethylenediaminetetraacetic acid (pH 7.0). The dialyzed solution was treated with calcium phosphate gel (3 mg per mg of protein); after gentle shaking at 4° for 30 minutes, the gel was centrifuged and washed successively with 30 ml of 0.005 M 2-mercaptoethanol, 30 ml. of 0.05 M potassium phosphate buffer (pH 6.6) containing 0.005 M 2-mercaptoethanol, and (three times) with 30 ml. of 0.1 M potassium phosphate buffer (pH 6.6) containing 0.005 M 2-mercaptoethanol. The enzyme was eluted with 30 ml of 0.5 M potassium phosphate buffer containing 0.005 M 2-mercaptoethanol.

STEP 8. FRACTIONATION WITH AMMONIUM SULFATE.—The solution obtained in the preceding step was treated with saturated ammonium sulfate (adjusted to pH 6.6 with ammonium hydroxide) containing 0.005 M 2-mercaptoethanol in a concentration equivalent to 0.25 saturation. After 30 minutes of stirring, the precipitate was removed by centrifugation at 17,000 $\times$ *g* for 15 minutes. The supernatant solution was adjusted to 0.5 saturation with respect to ammonium sulfate, and the mixture was stirred for 30 minutes and then centrifuged. The precipitate was dissolved in 2 ml of 0.02 M potassium phosphate buffer (pH 7.30) or 0.02 M 2-amino-2-(hydroxymethyl)-1, 3-propanediol–HCl buffer (pH 7.30) containing 0.15 M NaCl and 0.005 M 2-mercaptoethanol, and then dialyzed at 4° for 18 hours against two changes of 500 ml each of the same buffer.

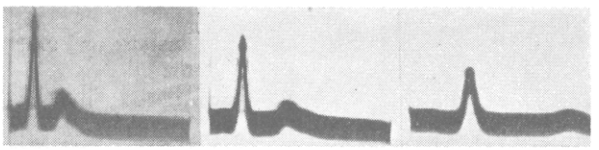

FIG. 1.—Electrophoretic diagrams (descending) of the enzyme; see text, step 9 of the isolation procedure. Protein concentration, 0.83%; 0.02 M 2-amino-2-(hydroxymethyl)-1,3-propanediol–HCl buffer containing 0.15 M NaCl and 0.005 M 2-mercaptoethanol (pH 7.30); temperature, 2°; 15 mamp. The photographs were taken after 5580 (*left*), 9420 (*center*), and 14,340 (*right*) seconds. Calcium phosphate gel was used; the final preparation was obtained after a series of compensations over an additional 4-hour period.

STEP 9. ELECTROPHORESIS.—Electrophoresis of the dialyzed solution was carried out in a Perkin-Elmer Model 38A Tiselius apparatus, in a 2-ml cell. A protein component representing about 30% of the total protein moved toward the anode more rapidly than did the enzyme (Fig. 1). By successive compensation over a period of 6 to 8 hours, the enzyme was obtained free of this impurity and was withdrawn from the cell with a blunt 20-gauge hypodermic needle. Additional enzyme (approximately 8–10 mg for a preparation from 2400 g of sheep brain) was obtained by re-electrophoresis of the impurity.

The following procedure was employed to remove ammonia and inorganic phosphate from the enzyme preparation. The enzyme (15 mg per 2 ml) was mixed with 0.4 ml of a solution containing 5 μmoles of L-glutamate, 1 μmole of ATP, and 5 μmoles of $MgCl_2$ (adjusted to pH 7.2), and was then incubated at 37° for 10 minutes. The solution was then dialyzed at 4° for 24 hours against four changes of 1000 ml each of 0.005 M 2-mercaptoethanol containing 0.001 M ethylenediaminetetraacetic acid (pH 7.2). About 5 g of Permutit was added to the external solution at the start of the final dialysis.

COMMENTS ON THE ISOLATION PROCEDURE.—A number of attempts were made to isolate the enzyme in the absence of 2-mercaptoethanol. Although active enzyme was obtained at each step, the specific acitivity was invariably lower than that observed when the mercaptan was present.

In step 3, it is important to provide efficient cooling and mixing during acidification, and in step 4 similar precautions are advisable during adjustment of pH by addition of alkali. If these precautions are not observed, considerable activity may be lost.

In step 6, it was found to be important to bring the pH to exactly 4.8 for the first precipitation; if the pH was lower than this value, much activity was lost.

The enzyme has also been purified by a modified procedure in which alumina C_γ gel was used in place of calcium phosphate gel. Alumina C_γ gel, as a suspension of 20 mg per ml, was added to the solution obtained in step 7 prior to dialysis (2 mg of gel per mg of protein), and the mixture was shaken gently for 45 minutes. The gel was centrifuged at 9,000 $\times$ *g* for 15 minutes and washed

TABLE I
SUMMARY OF PURIFICATION OF THE ENZYME[a]

Step	Volume (ml)	Total Protein (mg)	Protein Concentration (mg/ml)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)
Step 1, homogenate	2,400	220,000	91.5	9,740	0.044	
Step 2, extract	3,080	39,100	12.7	92,500	2.37	[100]
Step 4, extract	380	6,010	15.8	51,700	8.60	56
Step 5	430	2,360	5.49	40,700	17.2	44
Step 7, after dialysis	25	628	25.1	28,700	45.7	31
Step 7, after elution	30	264	8.81	18,500	70.0	20
Step 8	3.2	138	43.1	12,000	87.0	13
Step 9	3.0	44.1	14.7	6,700	152	7.2

[a] From 2400 g of fresh sheep brain.

successively with two portions of 40 ml of 0.25 M potassium phosphate buffer (pH 6.6) containing 0.005 M 2-mercaptoethanol. The enzyme was eluted with 40 ml of 0.5 M potassium phosphate buffer (pH 6.6) containing 0.005 M 2-mercaptoethanol. Fractionation with ammonium sulfate and electrophoresis were carried out as described above (steps 8 and 9). When alumina C_γ gel was employed, the impurity found on electrophoresis represented about 10% of the total protein.

A summary of a representative isolation of the enzyme is given in Table I. The apparent over-all purification (from homogenate to final preparation) given in Table I is about 3400-fold. However, we encountered considerable variability in the activity values of homogenates, a finding which may be due to inhibition of the enzyme by other components of the homogenate, or to the presence of variable amounts of ATP-ase. For this reason, the exact over-all purification cannot be calculated. Although the specific activity values for the initial homogenates varied greatly, the values obtained for the final preparations were close to 150. The yields varied between 20 and 50 mg from 2400 g of sheep brain. Assuming that 80% of the enzyme is recovered in step 2 and that the final preparation is pure, it may be calculated that the enzyme represents about 0.3% of the brain protein.

The enzyme was not active when ADP (at several concentrations) was substituted for ATP in the assay. The enzyme exhibited no ATP-ase activity, as determined by its action on ATP^{32}.

Ultraviolet Absorption of the Enzyme.—The purified enzyme exhibited a single absorption maximum at 280 mμ; the absorbancy at 280 mμ was 1.00 for a solution containing 1 g per liter of protein. The ratio of the absorbancy at 280 mμ to that at 260 mμ was 1.70. Calculation of the protein concentration from absorbancy data (Layne, 1957) gave a value that was within 5% of the value obtained by the procedure of Lowry *et al.* (1951). The enzyme contained 12.6% nitrogen as determined by the Kjeldahl procedure.

Electrophoresis and Ultracentrifugation Studies.—Electrophoresis of the enzyme at pH values of 6.60, 7.28, and 7.86 revealed single symmetrical peaks in each case. Representative patterns are given in Figure 2. The corresponding mobilities were -1.51×10^{-5}, -1.91×10^{-5}, and -2.22×10^{-5} cm^2 $volt^{-1}$ $sec.^{-1}$. Extrapolation of the mobility values to zero mobility gave an apparent isoelectric point of about 4.2; this value is close to the point of minimum solubility (see step 6 of the isolation procedure).

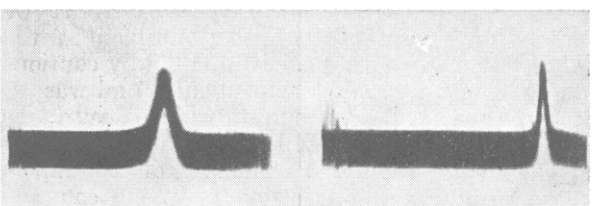

FIG. 2.—Electrophoretic diagrams (ascending) of the purified enzyme. Protein concentration, 0.5%; 0.02 M buffer as described in Figure 1; temperature 2°; 17 mamp. *Left*, pH 6.60; 17,100 seconds. *Right*, pH 7.86; 3,625 seconds.

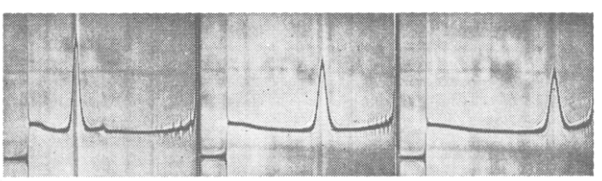

FIG. 3.—Sedimentation diagrams of the purified enzyme. Protein concentration, 1.44%; 0.02 M potassium phosphate buffer containing 0.15 M NaCl and 0.005 M 2-mercaptoethanol (pH 7.10); temperature, 4°; bar angle, 70°; speed, 59,780 rpm; the photographs were taken 16, 40, and 56 minutes after final speed was reached; sedimentation is from left to right.

When the enzyme was examined in the ultracentrifuge about 95% of the protein sedimented as a single symmetrical component (Fig. 3). Two very small components were observed; one of these sedimented more rapidly and the other less rapidly than did the major component. The heavier component may represent an aggregated form of the protein. The lighter material exhibited about the same sedimentation properties as does an enzymatically inactive degradation product of the enzyme obtained by treatment with sodium dodecyl sulfate.[1] Ultracentrifuge studies carried out in the separation cell of Yphantis and Waugh (1956) indicated that all of the enzyme was sedimented to the lower section of the cell within 2 hours at 59,780 rpm under the conditions given in Figure 3. Four separate determinations of the sedimentation coefficient, carried out under the conditions described in Figure 3, gave values of 14.8, 15.1, 15.1, and 14.5 *s*, with protein concentrations, respectively, of 0.97, 0.68, 0.96, and 1.44%. Molecular weight

[1] Further studies on the physical properties of the enzyme, carried out in collaboration with Dr. Rudy H. Haschemeyer, are in progress and will be reported in a subsequent publication.

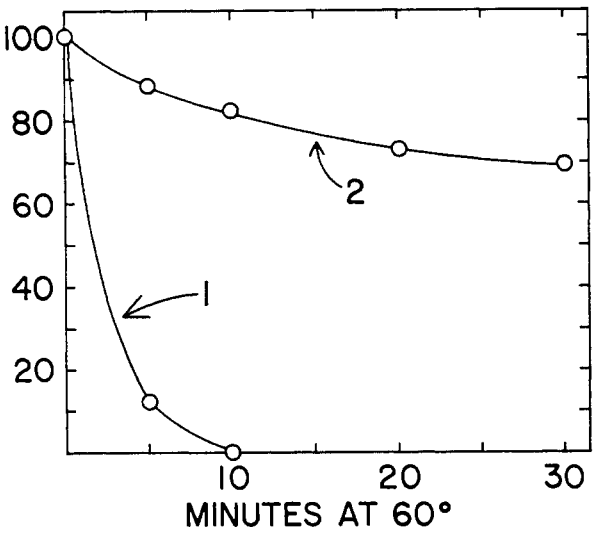

FIG. 4.—Protection of the enzyme against thermal denaturation. The enzyme (0.156 mg of the preparation obtained in step 4 of the isolation procedure) in a final volume of 1 ml containing 50 μmoles of imidazole-HCl buffer (pH 7.20), 25 μmoles of 2-mercaptoethanol, 10 μmoles of ATP, and 50 μmoles of magnesium chloride, was heated at 60° (curve 2). In curve 1, ATP and magnesium chloride were omitted. Similar results were obtained when either ATP or Mg^{++} was omitted. After heating, the mixtures were quickly cooled in ice, and enzymatic activity was determined after addition of 0.5 ml of a solution containing 50 μmoles of L-glutamate and 100 μmoles of hydroxylamine. The ordinate represents percentage of initial activity.

determinations by the procedures of Ehrenberg (1957) and Klainer and Kegeles (1955) gave values of 430,000 and 497,000 respectively.

Protection of the Enzyme Against Thermal Denaturation.—In an effort to find a suitable procedure for the isolation of the enzyme, a systematic study of the heat denaturation of the enzyme at step 4 of the procedure was carried out. In the presence of imidazole-HCl buffer (pH 7.2) alone, about 15% of the activity was lost at 55° in 40 minutes, while virtually all of the activity disappeared in 10 minutes at 60°. Subsequent study revealed that 0.01 M ATP plus 0.05 M $MgCl_2$ protected the enzyme significantly at 60° (Fig. 4). Protection required both ATP and Mg^{++}; AMP, CTP, ITP, UTP, and GTP were not effective in the presence or absence of $MgCl_2$. Addition of the following (in 0.01 M concentrations) to the enzyme in imidazole-HCl buffer (pH 7.2) was also ineffective in protecting the enzyme (*i.e.*, the results were identical to the result shown in curve 1, Figure 4): ATP and L-glutamate; L-glutamate; L-glutamate and Mg^{++}; NH_4Cl and Mg^{++}; AMP, Mg^{++}, and L-glutamate; L-glutamine; L-glutamine and ADP; L-glutamine and Mg^{++}; phosphate; phosphate and Mg^{++}; phosphate, Mg^{++}, and glutamate.

When ADP was used in place of ATP under the conditions described in Figure 4, about 70% of the activity remained after 10 minutes at 60°; in these studies the enzyme was dialyzed at 4° after heating to remove ADP, which inhibits enzymatic activity. Variation of the concentration of ATP (with a constant ATP:Mg^{++} ratio of 1:5) revealed that the concentration of ATP required for half-maximal protection of the enzyme was 0.001 M. Similar results were obtained with an ATP:Mg^{++} ratio of 1:2. The purified enzyme was also protected from thermal denaturation by ATP plus Mg^{++}; however, the purified enzyme was less stable than the preparation obtained in step 4, and only about 50% of the initial activity remained after 30 minutes at 60°.

Effect of Concentration of Substrates on Activity.—The effect of varying the concentrations of L- or D-glutamate, NH_4^+, NH_2OH, and ATP on enzyme activity was studied. The conditions employed in these experiments, and the results expressed in terms of apparent K_m values, obtained by the method of Lineweaver and Burk (1934), are given in Table II.

TABLE II
APPARENT KM VALUES

Substrate	K_m	Conditions[a]
L-glutamate	2.5×10^{-3}	NH_4^+
D-glutamate	1.5×10^{-2}	NH_4^+
L-glutamate	2.5×10^{-3}	NH_2OH
D-glutamate	1.9×10^{-3}	NH_2OH
NH_4^+	1.8×10^{-4}	L-glutamate
NH_4^+	1.4×10^{-2}	D-glutamate
NH_2OH	1.5×10^{-4}	L-glutamate
NH_2OH	3.5×10^{-3}	D-glutamate
ATP	2.3×10^{-3}	L or D-glutamate; NH_2OH

[a] The reaction mixtures contained initially 50 μmoles of imidazole-HCl buffer (pH 7.20), 25 μmoles of 2-mercaptoethanol, 0.2 to 1.2 units of enzyme, 50 μmoles of glutamate, 100 μmoles of NH_4Cl or NH_2OH, 10 μmoles of ATP, and 20 μmoles of $MgCl_2$ in a final volume of 1 ml; temperature 37°.

DISCUSSION

The present procedure appears to yield the most highly purified preparation of the enzyme thus far reported; the virtual absence of ammonia, inorganic phosphate, ATP-ase, and adenylate kinase render it suitable for studies on the mechanism of the reaction. The yield of enzyme is relatively good; in our laboratory we have found that a single individual can readily carry out the isolation of about 40 mg of enzyme in 4 days. Previous reports on the purification of the enzyme include a 19-fold purified preparation from sheep brain (Elliott, 1951), preparations of about 10-fold purification from pigeon liver (Speck, 1949), and a preparation from peas described by Elliott (1953) that was reported to be 1000- to 2000-fold purified. The last-mentioned preparation, which has been used in a number of laboratories, was reported to exhibit one main component and two smaller ones on electrophoresis, and to exhibit two approximately equal components in the analytical ultracentrifuge (Elliott, 1953). Levintow *et al.* (1955) examined a preparation of the pea enzyme (prepared by a modification of Elliott's procedure) in the ultracentrifuge; the enzyme activity sedimented with a component that represented about 20% of the total protein and exhibited a sedimentation coefficient of 13.9 *s*. It is of interest that this value is not far from the values of the sedimentation coefficient observed for the present enzyme preparation. Varner and Webster (1955) described another modification of Elliott's procedure, which

was purported to give a preparation that was 4100-fold purified; physical characterization was not reported. In the experience of the present authors, relative purification values for this enzyme, whether obtained from peas or sheep brain, are unreliable guides to purity because of the extremely low and variable activities of the initial homogenates. It is probable that adenylate kinase and ATP-ase present in the initial homogenates influence the observed activity values.

The protection of the enzyme against thermal denaturation at 60° by ATP plus Mg^{++} is of interest, not only because this fact is useful in the isolation of the enzyme but also because this observation suggests that ATP is capable of combining with the enzyme. Such combination apparently requires Mg^{++}, but takes place in the absence of glutamate. Considerations discussed elsewhere (Meister, 1961) suggest that combination of ATP with the enzyme is the first step in glutamine synthesis; and previous studies (Krishnaswamy *et al.*, 1960; Meister *et al.*, 1961) indicate that cleavage of ATP does not occur in the absence of glutamate. The concentration of ATP required for half-maximal protection of the enzyme (at step 5 of the isolation procedure) is about 0.001 M, a value which is of the same order of magnitude as the apparent K_m value for ATP in the catalytic reaction (Table II). The other values given in Table II are consistent with earlier studies in which the enzyme from peas was used.[2] It is of particular interest that the apparent K_m value for D-glutamate with NH_4^+ is significantly higher than that for D-glutamate with NH_2OH and those for L-glutamate. Furthermore, the K_m value for NH_4^+ with D-glutamate is much larger than that for NH_4^+ with L-glutamate. These results are consistent with the suggestion made previously (Levintow and Meister, 1953) that the synthesis of glutamine involves a relatively optically nonspecific activation step followed by a more optically specific reaction of ammonia with the activated glutamate intermediate. Studies on the mechanism of glutamine synthesis in which the present enzyme preparation has been employed will be described in a subsequent communication.

[2] Dr. Carl Monder in this laboratory obtained the following K_m values for the pea enzyme (pH 7.5; 37°): 3.0×10^{-3} (L-glutamate, with NH_4^+), 5.5×10^{-2} (D-glutamate, with NH_4^+), 4.5×10^{-3} (L-glutamate, with NH_2OH), 5.5×10^{-3} (D-glutamate, with NH_2OH), 1.3×10^{-5} (NH_4^+ with L-glutamate), 2×10^{-2} (NH_4^+, with D-glutamate), 6.3×10^{-5} (NH_2OH, with L-glutamate), 4.1×10^{-4} (NH_2OH, with D-glutamate). Other values reported in the literature for the enzyme from peas include those of Varner (1960).

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