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STUDIES ON THE ENZYMATIC SYNTHESIS OF GLUTAMINE[1,2]

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An enzyme catalyzing the following overall reaction

1. Glutamate + NH_3 + ATP $\overset{Mg^{++}}{\rightleftharpoons}$ Glutamine + ADP + Pi

has been shown to occur generally in plants (7, 8, 25), animals (7, 21) and bacteria (11). If either hydroxylamine or hydrazine is substituted for NH_3, the enzyme forms glutamyl hydroxamate or glutamyl hydrazide at rates about equal to those for glutamine formation. This enzyme was isolated by Elliott in a highly purified form from dried green peas and some of its properties described (8). The possibility that the glutamine synthesizing enzyme may be identical with the enzyme which catalyzes the transfer reactions described by Stumpf and Loomis (22)

2. $GluNH_2 + NH_2OH \longrightarrow GluNHOH + NH_3$

3. $GluNH_2 + N^{15}H_3 \rightleftharpoons GluN^{15}H_2 + NH_3$

has already been considered both by Elliott (8) and by Levintow and Meister (14).

Although the enzyme from green peas has been highly purified by Elliott (8), a number of its important properties have not been reported. Furthermore, the enzyme in its highest state of purity as obtained by the procedure of Elliott is somewhat unstable. We have improved and extended Elliott's purification procedure and obtained an enzyme purified some 4000-fold over the original extract. Data concerning the stability of the enzyme from green peas and its affinity for glutamate, ATP and hydroxylamine have been obtained and are reported here for the first time. Information concerning the specificity of the enzyme and the equilibrium constant of the overall reaction has been determined. These data, when taken with those of Elliott (8), serve to characterize rather completely the nature of the pea enzyme catalyzing the synthesis of glutamine. In addition, further properties of the transfer activities of this system and of the arsenate-activated hydrolysis of glutamine are presented. Finally, data have been obtained by exchange studies with radioactive phosphate and radioactive glutamate which have a bearing on the mechanism of glutamine synthesis and its relation to the transfer activities of the enzyme.

EXPERIMENTAL METHODS

PURIFICATION OF THE ENZYME: The enzyme was extracted from dried green peas, and purified according to the first four steps in the procedure of Elliott (8) (through the first nucleic acid precipitation and extraction). We found the "inhibitor" mentioned by Eliott (8) and partly removed by protamine treatment to be an ATP hydrolase activity and not a true inhibitor. The final steps of the purification procedure reduce this activity to the point where it does not interfere with the glutamine synthesis reaction. The extract from the first nucleic acid fractionation usually contains some (up to 60 % of the total protein) high molecular weight protein which can be removed by centrifugation at 140,000 × g. Removal of this material is essential if one is to obtain a consistent and effective ammonium sulfate fractionation. The fractionation with ammonium sulfate, dialysis and fractionation with nucleic acid were according to the procedure of Elliott (7).

The preparation from the second nucleic acid fractionation (about 1 % protein in 0.05 M phosphate buffer at pH 7.3) was further fractionated by the addition of 0.20 volume of 95 % ethanol. The ethanol was precooled to − 20° C and the centrifugation made at − 5° C. The inactive precipitate was discarded. The supernatant had a specific activity 4 times that of the starting preparation and represented a 4100-fold increase in specific activity over the original extract. This ethanol fractionation is useful as an additional purification step since it removes inactivated enzyme as well as other proteins. The resulting

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[4] Abbreviations used: Glu, glutamic acid; ATP, adenosinetriphosphate; $GluNH_2$, glutamine; ADP, adenosinediphosphate; Pi, orthophosphate; GluNHOH, glutamylhydroxamate; E, enzyme, TRIS, tris-(hydroxymethyl)-aminomethane; PCMB, *p*-chloromercuribenzoate; Pi^{32}, radioactive orthophosphate; ADP^{32}, radioactive phosphate labeled ADP; ATP^{32}, radioactive phosphate labeled ATP; GSH, glutathione; PPi, inorganic pyrophosphate; AMP, adenosinemonophosphate; As, arsenate; As^{76}, radioactive arsenate.

ethanol solution of the enzyme, although rather dilute, can be kept in solution at $-20°$ C for at least one month without any loss in activity. The enzyme can be precipitated from this solution by increasing the ethanol concentration to 50 %.

ASSAY: Standard assay conditions for the synthetic activity were as follows: 45 micromoles TRIS buffer (pH 7.8), 50 micromoles potassium glutamate, 2 micromoles ATP, 30 micromoles $MgSO_4$, 10 micromoles β-mercaptoethanol, and 40 micromoles hydroxylamine, per ml of reaction mixture (usually 4 ml). Enough enzyme was added to produce about 0.25 micromole of glutamylhydroxamate per ml in 15-minute incubation at 35° C. The color of the ferric-hydroxamate complex (15) was read on a Klett-Summerson colorimeter with a 540 mμ filter. One unit of activity corresponds to that quantity of enzyme catalyzing the formation of 1 micromole of glutamylhydroxamate per hour under the standard test conditions.

ATP-hydrolase activity was determined by incubating 0.5 ml of enzyme preparation with 0.3 ml of 0.01 M TRIS (pH 7.4) containing 0.001 M $CaCl_2$, and 0.2 ml of 0.01 M ATP for 30 minutes at 25° C (9). The amount of inorganic phosphate enzymatically liberated was then determined.

Standard assay conditions for measurement of transfer activity were as follows: 45 micromoles TRIS buffer (pH 7.4), 30 micromoles $MgSO_4$, 10 micromoles β-mercaptoethanol, 0.5 micromole ADP, 50 micromoles glutamine, 25 micromoles inorganic phosphate, 40 micromoles hydroxylamine per ml of reaction mixture. Enough enzyme was added to produce about 0.25 micromole of glutamyl-hydroxamate per ml in 15 minutes at 35° C.

The hydrolysis of glutamine in the presence of arsenate and phosphate was followed by chromatographing a 3 λ spot of each reaction mixture with 88 % phenol, developing the color with ninhydrin and reading the maximum intensity of each spot according to the method of Block (3). Absolute values were determined by running a series of glutamine and glutamic acid standards under the same conditions.

Phosphate labeled ADP was prepared by incubation of Pi^{32} and ATP with fresh sheep heart homogenate according to the method of Hems and Bartley (10). Labeled ADP and ATP were separated from the incubation mixture by paper chromatography according to Bandurski and Axelrod (2). The labeled phosphate compounds resulting from the exchange experiments were also separated by paper chromatography, the spots located with molybdate reagent, cut out, and counted with a 1.4 mg/cm^2 end window tube and a standard scaling circuit.

RESULTS

THE SYNTHESIS REACTION, *Stability of Enzyme:* The stability of the enzyme is indicated by the maintenance of full activity of dilute ethanolic solutions of the enzyme for periods up to one month at $-20°$ C. As a matter of practical interest in the consideration of possible purification procedures, the pH stability of the enzyme was determined (fig. 1). At room temperature it is markedly unstable particularly below pH 6, in periods as short as one hour.

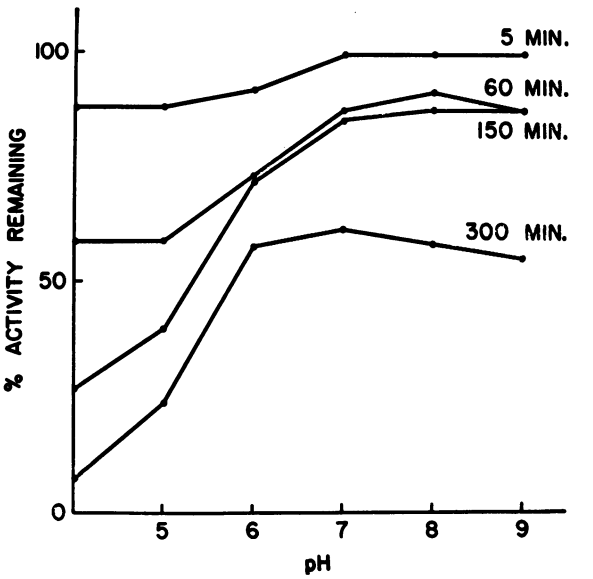

FIG. 1. The effect of pH on the stability of the glutamine synthesizing enzyme. Each sample was preincubated at 25° C at the pH values shown.

Equilibrium: The equilibrium constant of the synthesis reaction was determined by approaching the equilibrium from the glutamine side and measuring the net synthesis of ATP from ADP and radioactive inorganic phosphate. Figure 2 shows the data obtained in one of these experiments. The equilibrium constant calculated for the forward reaction at pH 7.4 is 1.7×10^3. It can be calculated from the equilibrium

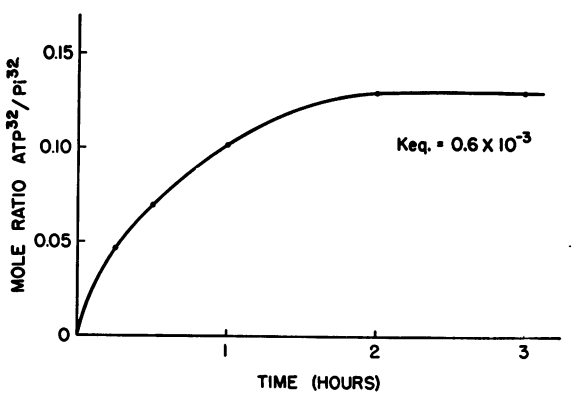

FIG. 2. Evidence for the reversibility of the glutamine synthesis reaction. Each sample contained: 45 micromoles TRIS buffer (pH 7.4), 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 2 micromoles ADP, 50 micromoles glutamine, 5 micromoles of Pi^{32}, 27 units of enzyme/ml of reaction mixture. Temperature of incubation, 35° C. Synthesis of ATP is expressed as mole ratio of ATP formed to orthophosphate conc at the time ATP conc is measured.

value that the free energy of formation of the γ-glutamylamide bond is about 4,800 calories less than for the pyrophosphate of ATP. This surprisingly small difference may indicate that the amide bond of glutamine has a higher free energy of formation (3,500 cal) than the amide bond of asparagine (4), or it may indicate that the commonly accepted value of 10,500 cal for the ΔF of the formation of the terminal phosphate linkage of ATP is too high. Levintow and Meister (14) have recently determined this same equilibrium constant by a different method and arrived at a value of 1.2×10^3 at pH 7.0 and 37° C. They make the assumption that the ΔF of formation of the amide bond of glutamine is the same as that for asparagine and suggest that the true ΔF of formation of ATP from ADP and Pi is near 8,000 cal. An independent confirmation of this comes from the calculations of Anfinsen and Kielley (1).

Properties of the Enzyme: Although Elliott (7) has determined the reactant affinities of the enzyme from sheep brain, the same data have not previously been reported for the enzyme from peas. The initial rates of the synthetic reaction as a function of the concentration of glutamate, ATP, and hydroxylamine are shown in figure 3. The relatively high affinity of the enzyme for hydroxylamine suggests the usefulness of this system as a trapping system for low concentrations of hydroxylamine. It is noteworthy that potassium could not be shown to be required for the synthesis of glutamine. This is in contrast to the potassium requirement for the synthesis of glutathione (19) and of γ-glutamylcysteine (26).

Although iodoacetate and oxidized glutathione in concentrations up to 1×10^{-2} M and an excess of solid

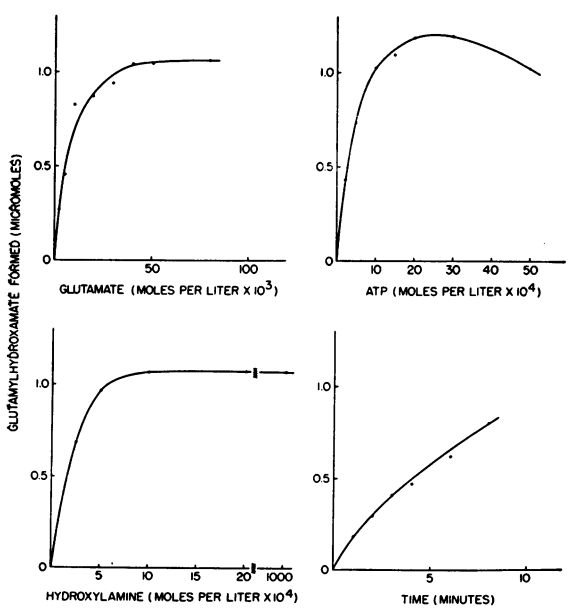

FIG. 3. The rate of the synthesis reaction as a function of the concentration of reactants, and as a function of time. Assay conditions were as already described with the exception of the variable being studied.

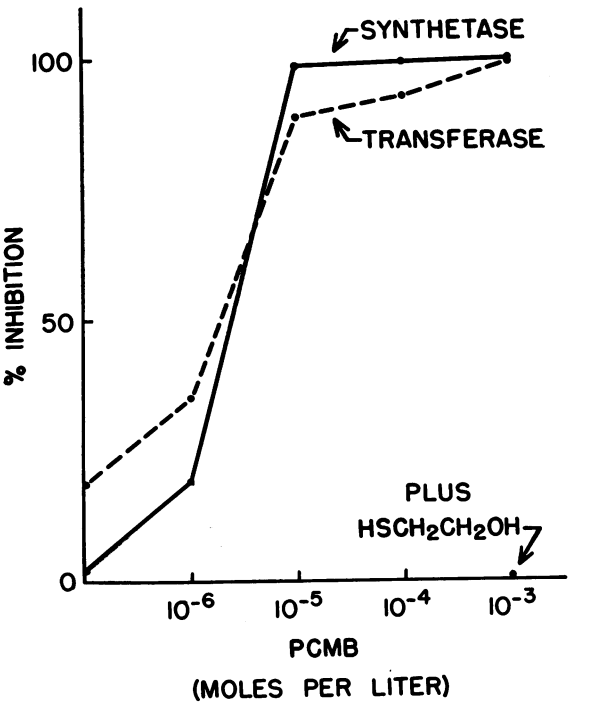

FIG. 4. Para-chloromercuribenzoate as an inhibitor of the transfer and synthesis reactions. Experimental conditions were identical to the standard assay procedures, with the exception that β-mercaptoethanol was omitted except where noted.

cystine all fail to inhibit the synthetic activity of the enzyme, 1×10^{-3} M PCMB completely inhibits it (8). Figure 4 shows the dependence of the inhibition of glutamine synthetase on PCMB concentration. This inhibition is completely reversed by β-mercaptoethanol. These facts indicate the participation of at least one sulfhydryl group during the reaction and demonstrate that this group (or groups) is not readily available for reaction with all sulfhydryl reagents.

Table I presents additional data demonstrating the inhibitory effects of the end products ADP and Pi.

TABLE I

EFFECT OF ADP, PHOSPHATE, ARSENATE AND ARSENITE ON THE RATE OF GLUTAMYLHYDROXAMATE SYNTHESIS

SYSTEM	GLUNHOH	INHIBITION
	micromoles	%
Complete	1.00	..
+ADP (1 micromole/ml)	0.75	25
+Phosphate (12 micromoles/ml)	0.55	45
+ADP and phosphate	0.41	59
+Arsenate (12 micromoles/ml)	1.22	22 % stimulation
+Arsenite (12 micromoles/ml)	0.92	8

The complete system components were the same as those described for the standard assay.

The arsenate effect on the synthetic reaction is especially interesting as arsenate has already been shown (23) to enhance markedly glutamyl transfer activity.

The purified enzyme would not catalyze the formation of hydroxamates of any of the folowing amino acids: aspartic acid, glycine, alanine, β-alanine, arginine, valine, leucine, cysteine or lysine. Aspartic acid and glycine inhibited the formation of glutamylhydroxamate. A concentration of glycine or aspartate nine times that of the glutamate, inhibited glutamylhydroxamate formation by 50 %. The enzyme does catalyze the formation of D- and L-α-amino-adipylhydroxamates at a rate of about 1/20 that for the formation of glutamylhydroxamate (12).

In a preliminary report (30) we suggested a reaction mechanism for the enzymatic synthesis of glutamine which involved (a) phosphorylation of the enzyme by ATP as a first step, (b) reaction of the enzyme-phosphate with glutamate to form an enzyme-glutamate complex, and finally (c) reaction of the enzyme-glutamate complex with ammonia or hydroxylamine to form the amide or hydroxamate. It was also suggested that the transfer reaction involved formation of an enzyme-glutamate intermediate from glutamine and enzyme, followed by reaction of the intermediate with ammonia or hydroxylamine.

In light of the fact that the transfer reaction (reaction c above) requires ADP and inorganic phosphate or arsenate (as well as magnesium or manganous ions) (8), one might expect reaction c to be rate-limiting in the overall synthetic reaction at the start of the reaction when there is no ADP or inorganic phosphate present. If this were true, the synthetic reaction in the initial stages should have the kinetics of an autocatalytic reaction. This is, however, not the case, as the data presented in figure 3 show. These facts suggest that the mechanism postulated earlier needs further elaboration, even though it does satisfactorily account for the fact that intermediates such as glutamylphosphate have not been found. That the mechanism proposed is incomplete is evidenced too, by the fact that it does not account for the ADP, Pi or arsenate requirement for the transfer reaction. This reaction has, therefore, been studied in detail.

THE TRANSFER REACTION: Enzyme systems which catalyze the exchange of the amide group of L-glutamine for either ammonia or hydroxylamine (reactions 2 and 3) have been shown to have a widespread occurrence in plants (6, 8, 22, 23), animals and bacteria (11, 24). The systems extracted from plant and animal tissues appear to have identical cofactor requirements, these being manganous or magnesium ions, ADP or ATP and phosphate or arsenate. Bacterial extracts may contain at least two different systems which catalyze reactions 2 and 3. One system apparently has no cofactor requirement although its activity is enhanced by cupric ions (24). The second system requires manganous ions, phosphate or arsenate and possibly ADP (24).

The ratio of the synthetic activity to the transfer activity of the enzyme preparations was checked at each stage in the purification procedure and, in confirmation of Elliott's results (8) was found to be constant throughout the purification. As with the synthetic activity, 1×10^{-2} M iodoacetate and 1×10^{-2} M oxidized glutathione were ineffective as inhibitors. However, *p*-chloromercuribenzoate was as effective as an inhibitor of the transfer reaction as of the syn-

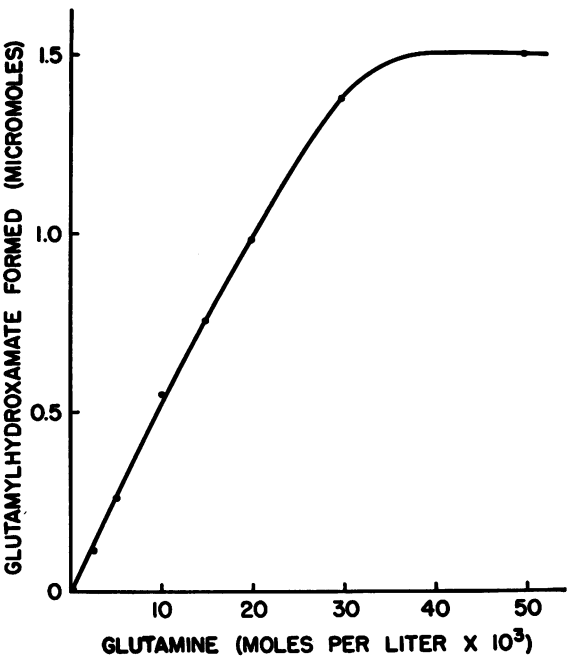

FIG. 5. Rate of the transfer reaction as a function of glutamine conc. Experimental conditions identical to the standard assay procedure, with the exception of the glutamine conc.

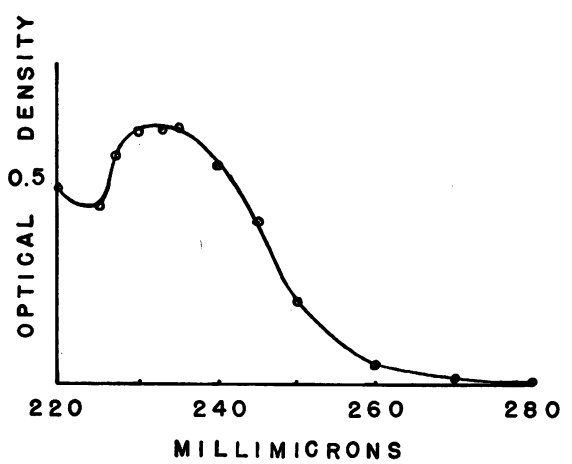

FIG. 6. Difference spectrum indicating complex formation between glutamine synthetase and PCMB. One cuvette contained 0.1 mg purified enzyme in 3.10 ml, the second cuvette contained 0.1 mg purified enzyme and 100 micromoles of PCMB, the third cuvette (blank) contained 100 micromoles of PCMB.

thetic reaction. β-Mercaptoethanol completely reversed both inhibitions. Figure 4 compares the inhibition of the two reactions as a function of the concentration of PCMB. The differences in the degree of inhibition are small, but real. The general similarity of the inhibition curves suggests that the same sulfhydryl group (or groups) may be involved in the two catalytic functions. We have already suggested (30) that some enzyme-bound intermediate may be common to the two reactions.

With the thought in mind that one of the enzyme-bound intermediates in the synthesis reaction and transfer reaction might be an enzyme-thiol ester, an attempt was made to find optical evidence (9) for such a linkage. Using a Beckman DU spectrophotometer with the Beckman photomultiplier attachment, no evidence for such a linkage could be found although a peak at 235 mμ for the complex formation of the enzyme with PCMB (5) was readily observable (fig 6).

The small difference in the degree of inhibition of the two activities may somehow result from the fact that in one case the enzyme-bound intermediate is formed by the approach of glutamate to the enzymatic site and in the other by the approach of glutamine (L-glutamine obtained from Nutritional Biochemicals Corporation). In connection with the fact that the syntheic reaction is less sensitive than the transfer reaction to PCMB at the lower concentrations, it is noteworthy that the affinity of glutamate for the enzyme (fig 2) is greater than that of glutamine (fig 5).

The relative effectiveness of ADP and ATP as cofactors from the transfer reaction is shown in figure 7. The results confirm those of Stumpf (23) who used a transfer system from pumpkin seedlings. Even though ATP preparations are effective it is by no means certain that it is ATP itself which has the activity (16). An ATP preparation containing about 6 % ADP would give the results shown in figure 7 if one assumes that ATP does not compete effectively with ADP for some two point attachment necessary to the activity, but is effective in a one point attachment which causes inhibition. Since the enzyme preparation itself has an ATP-hydrolase activity it would be very difficult to prevent the formation of ADP during a reaction in which ATP was added as a cofactor. However, an experiment designed to detect an increase in rate of the transfer reaction with time in the presence of ATP as a cofactor showed the rate to be constant from zero time, i.e., the rate does not increase as more ADP is liberated from ATP. AMP is ineffective as a cofactor in this reaction.

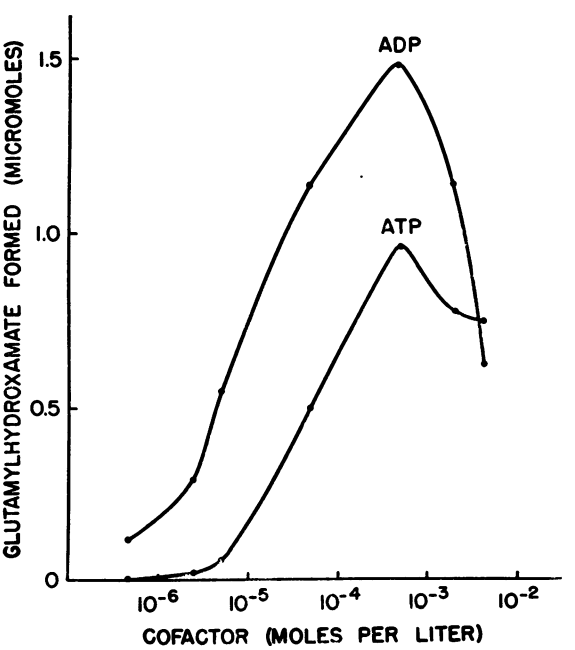

FIG. 7. ADP and ATP as cofactors in the transfer reaction. Experimental conditions were according to the standard assay procedure except for the variable being studied.

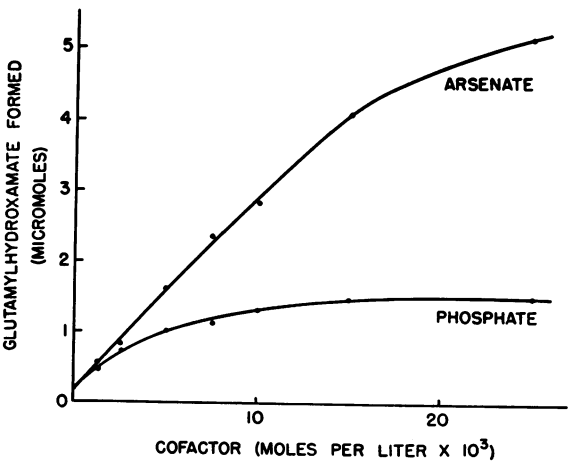

FIG. 8. Phosphate and arsenate as cofactors in the transfer reaction. Conditions were according to the standard assay procedure except for the variable being studied.

That arsenate is more effective than phosphate as a cofactor in the transfer reaction has been already noted by Stumpf et al (23). Figure 8 shows that this greater effectiveness holds true only at relatively high concentrations of phosphate and arsenate. Since the difference in the effectiveness of the two cofactors is very small at low concentration, it seems reasonable to suggest that the differences in the maximum potential effectiveness may also not be very great. In the case of phosphate this maximum is never realized because a high concentration of phosphate tends to drive the reaction toward the formation of glutamate, ATP and ammonia, possibly binding a part of the enzyme in the form of some phosphate-containing intermediate more stable than the one which reacts with hydroxylamine to form glutamylhydroxamate. The corresponding arsenate complex might be expected to be less stable, resulting in a higher concentration of enzyme available to carry out the transfer reaction

and, therefore, in an apparently greater effectiveness of arsenate as a cofactor. Arsenite is not effective as a cofactor in the transfer reaction but is only slightly inhibitory.

THE ARSENATE-ACTIVATED HYDROLYSIS: In addition to the transfer and the glutamine synthesis reactions, there is a third reaction catalyzed by this same enzyme preparation from peas. This is an arsenate-activated hydrolysis of glutamine (13, 14, 23). The reaction has an absolute requirement for ADP and magnesium or manganous ions (14) as well as for arsenate ions. It can be seen from the data in table II that the rate of the reaction is linear with time through the hydrolysis of 19 % of the glutamine present. ATP strongly inhibits the reaction. It is of interest to compare the production of glutamate when phosphate is substituted for arsenate. There is, of course, a little glutamate formed due to reversal of the synthesis reaction. When hexokinase and glucose

TABLE II

ARSENATE-ACTIVATED HYDROLYSIS OF GLUTAMINE BY GLUTAMINE SYNTHETASE FROM PEAS

CONDITIONS	TIME	GLUTAMATE FORMED
	min	*micromoles/ml*
Complete with arsenate	15	1.0
Complete with arsenate	30	2.2
Complete with arsenate	60	4.7
+ATP (0.5 micromoles/ml)	60	2.2
Complete with phosphate	60	0.02
+hexokinase and glucose ..	60	0.9

The complete system contained: 45 micromoles TRIS (pH 7.4), 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 1 micromole ADP, 25 micromoles arsenate or phosphate, 25 micromoles glutamine, and 70 units of enzyme per ml. Total reaction mixture was 4.0 ml. One mg of hexokinase (Pabst) and 7.2 mg of glucose were added where indicated.

are added to trap out the ATP as it is synthesized, there is a fairly rapid "hydrolysis" of glutamine. The rate of the reaction under these circumstances, however, is still only about 20 % of the rate of the arsenate-activated reaction. Because of the difference in rates of the arsenate system and the phosphate-hexokinase-glucose system, it appears that the step which is rate-limiting in the transfer reaction is also limiting in the hydrolysis reaction. It must be then that the arsenate-activated reaction proceeds rapidly because of the instability of some organic arsenate compound (or complex) formed during the reaction and which decomposes more rapidly than phosphate is transferred to ADP.

The degree of specificity of this enzyme with respect to the compounds which it activates for the transfer reaction has been noted by Stumpf (23) and by Levintow and Meister (14). The formation of glutamine from glutamylhydroxamate has not been detected even in the presence of a large excess of ammonium ions, indicating that the activation of glutamyl hydroxamate proceeds at a slow rate, as compared to glutamine activation.

From the results presented in table V, it appears that glutamate remains bound to the enzyme during the transfer reaction. The relation of this fact to mechanism proposals will be considered in the discussion section of this paper.

EXCHANGE REACTIONS: When the highly purified enzyme was used in exchange experiments similar to those reported earlier (30), it was found that there was essentially no exchange of Pi^{32} into ATP in the presence of glutamate unless a catalytic quantity of ammonia was simultaneously present.

Figure 9 shows the dependence of the exchange on the concentration of ammonium ions. That the exchange is not completely dependent upon the addition of ammonia is probably due to the presence of a small quantity of ammonia adsorbed on the enzyme. The earlier results (30) in which there was no apparent requirement for ammonia are probably due to the fact that the 270-fold purified enzyme still contained traces of ammonia. Also it is likely that the exchange experiments reported by Levintow and Meister (14), in which exchange of Pi^{32} into ATP was not depend-

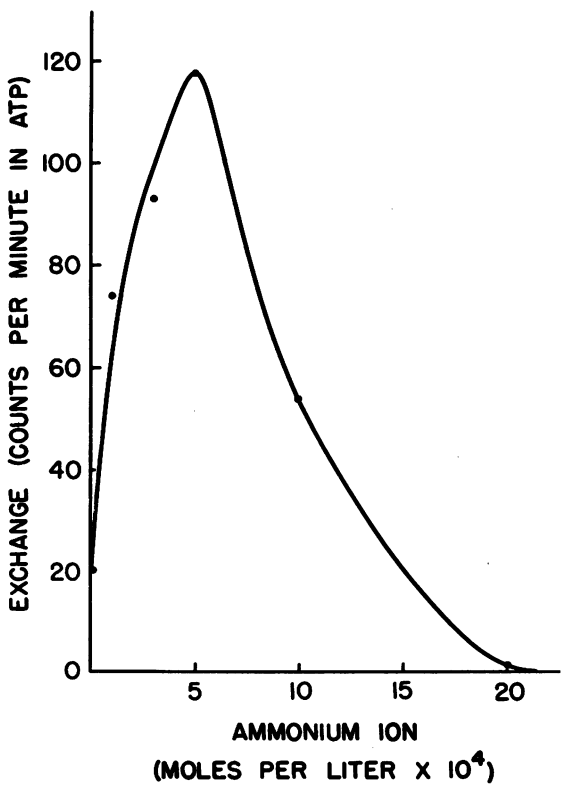

FIG. 9. The exchange of P^{32} into ATP as a function of ammonia conc. The complete system contained: 2.5 micromoles Pi^{32}, 2 micromoles ATP, 50 micromoles glutamate, 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 45 micromoles TRIS buffer (pH 7.4) and 40 units of enzyme/ml of reaction mixture. The incubation was at 35° C for 1 hr.

TABLE III

EXCHANGE OF Pi^{32} INTO ATP, CATALYZED BY PURIFIED GLUTAMINE SYNTHESIS

CONDITIONS	ATP	Pi
	cpm/10	*cpm/10*
Complete system	200	3600
-Ammonia	28	3800
-Ammonia + hydroxylamine	31	3800
Complete + PCMB to 10^{-6} M	170	3600
Complete + PCMB to 10^{-5} M	9	3800
Complete + PCMB to 10^{-5} M + β-mercaptoethanol	200	3600

The complete system contained: 2.5 micromoles Pi^{32} (380,000 cpm), 2 micromoles ATP, 50 micromoles glutamate, 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 45 micromoles TRIS buffer (pH 7.4), 12 micromoles ammonia (or hydroxylamine), 40 units of enzyme /ml of reaction mixture. Incubation was at 35° C for 1 hr.

ent upon added ammonia, can be explained on the basis of trace quantities of ammonia in their enzyme preparation. The measured exchange of Pi^{32} into ATP decreases to near zero at the higher concentrations of ammonia where all of the ATP initially present is used up.

The exchange of Pi into ATP, mediated by highly purified glutamine synthetase, did not occur unless all of the reactants and cofactors necessary for the overall synthesis reaction were present. Nor did exchange occur with catalytic quantities of hydroxylamine in place of ammonia. This is in agreement with the observation mentioned earlier that the rate of the transfer of the glutamyl radical from hydroxylamine to ammonia is very slow.

The data recorded in table III demonstrate that this exchange reaction is inhibited by the same levels of PCMB which are effective in inhibiting the synthetic reaction and the transfer reaction. Inhibition of the phosphate exchange reaction is also completely reversed by the addition of β-mercaptoethanol.

As illustrated by the data of table IV, exchange of ADP^{32} into ATP is also dependent upon the simultaneous presence of the full complement of reagents and cofactors necessary for the overall synthetic reaction. The rate of exchange of ADP^{32} into ATP equalled the rate of exchange of Pi^{32} into ATP.

TABLE IV

EXCHANGE OF ADP^{32} INTO ATP CATALYZED BY PURIFIED GLUTAMINE SYNTHETASE

CONDITIONS	ADP	ATP
	cpm/200	*cpm/200*
Complete system	174	226
-Ammonia	390	12
-Glutamate	400	7
-Ammonia and glutamate	400	15
-Enzyme	400	0

The complete system contained: 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 45 micromoles TRIS buffer (pH 7.4), 0.5 micromoles ADP^{32}, 2 micromoles ATP, 1.2 mocromoles ammonia, 50 micromoles glutamate, 2.5 micromoles Pi, and 40 units of enzyme per ml of reaction mixture. Incubation was at 35° C for 2 hrs.

In an exchange experiment in which D-glutamate replaced L-glutamate, the ratio of Pi^{32} exchanged into ATP in the presence of D-glutamate to that exchanged into ATP in the presence of L-glutamate was 10/280. Levintow and Meister (12) have reported that although the enzymatic synthesis of D-glutamine proceeds at about 40 % of the rate of the synthesis of L-glutamine, the rate of transfer of the glutamyl group from D-glutamine to hydroxylamine is only 3.93/286 of the rate of that transfer from L-glutamine. This similarity between the D-glutamate/L-glutamate ratios of the rates of the exchange reactions and the transfer reactions is further evidence that there can be no exchange of Pi^{32} or ADP^{32} into ATP except by the reversal of the overall synthetic reaction. The optical specificity of the enzyme which permits only a slow rate of formation of the hydroxamate from D-glutamine, also prevents a rapid exchange of Pi^{32} into ATP.

TABLE V

INCORPORATION OF GLUTAMATE-C-14 INTO GLUTAMINE DURING THE TRANSFER REACTION

SAMPLE	$GluNH_2$	GLU	GLUTAMATE EXCHANGED	GLUTAMATE TRANSFERRED
	cpm/ml	*cpm/ml*	*micromoles*	*micromoles*
Complete with phosphate	5,600	318,000	0.13	6.5
Complete with arsenate	2,000	340,000	0.04	9.0

The complete reaction mixture contained 30 micromoles Mg^{++}, 45 micromoles TRIS, 10^{-5} micromoles β-mercaptoethanol, 0.5 micromole ADP, 40 micromoles NH_4^+, 25 micromoles L-$GluNH_2$, 5 micromoles D, L-Glu-C^{14}, 5 micromoles L-Glu carrier and 12.5 micromoles Pi or As as indicated/ml of reaction mixture. Enough enzyme was added to each ml of reaction mixture to cause the formation of 6.5 micromoles of GluNHOH in the presence of Pi and 9.0 micromoles of GluNHOH in the presence of As, during the incubation period, when NH_2OH was substituted for NH_4^+.

Figure 10 shows the effect of arsenate on the apparent equilibrium of the glutamine synthesis reaction (approached from the glutamine side) as measured by incorporation of Pi^{32} into ATP. The possibility that the decrease of ATP^{32}/Pi^{32} in the presence of arsenate was due to the formation of an ADP-arsenate compound with an appreciable stability was considered and tested by incubating glutamine, ADP, magnesium and radioactive arsenate (As^{76}) with the enzyme. The reaction was stopped by cooling to 0° C, and an aliquot of the reaction mixture chromatographed at 0° C using the procedure of Bandurski and Axelrod (2). The chromatography required 4 hours, during which time the As^{76} spots, containing

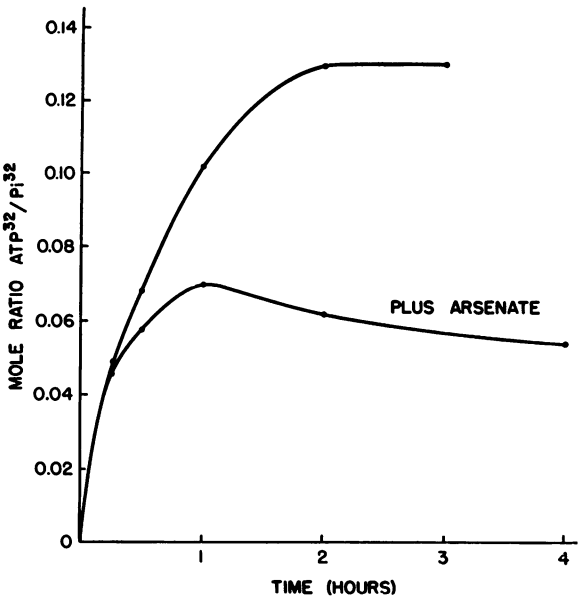

FIG. 10. The effect of arsenate on the equilibrium for the synthesis of glutamine. The incubation mixture contained: 45 micromoles TRIS buffer (pH 7.4), 30 micromoles Mg^{++}, 10 micromoles β-mercaptoethanol, 2 micromoles ADP, 50 micromoles glutamine, 5 micromoles of Pi^{32}, and 27 units of enzyme/ml of reaction mixture.

30,000 counts, moved about 5 inches. No radioactive spot other than that of inorganic arsenate could be detected and the arsenate spots were perfectly symmetrical with no indication of tailing which would suggest the presence of an arsenate ester which decomposed during the chromatographic development. From these data we have estimated that if ADP-arsenate is produced under the present conditions, it must have a half-life of less than 15 minutes. The fact that the ratio of ATP^{32}/Pi^{32} at equilibrium is much lower in the presence of arsenate is, therefore, a result of the arsenate-activated hydrolysis of glutamine, by which glutamate and ammonia are formed and the apparent equilibrium point shifted from that obtained in the absence of arsenate. The decrease of the ATP^{32}/Pi^{32} ratio with time (after 1 hour) is a further measure of this hydrolysis.

DISCUSSION

The data presented here, together with the results of other investigators, serve to characterize three primary catalytic activities of the glutamine synthesizing system and strongly suggest that these three activities are the manifestation of the properties of a single protein. This characterization is of importance because of the contribution which it makes to our appreciation of the complexity of the mechanism of the synthetic reaction. The manner in which this reaction proceeds has been the subject of speculation and experiment from the time of the earliest reports of Speck on the synthesis of glutamine. By analogy with other biosyntheses involving ATP, the reaction was first suggested to involve the formation of a γ-glutamyl phosphate intermediate which would then react with ammonia (perhaps non-enzymatically) to form glutamine. Vigorous efforts by several investigators failed, however, to show any direct evidence for such an intermediate.

The exchange experiments reported here indicate that the only intermediates formed during the synthesis of glutamine are enzyme-bound intermediates. It would appear that further exchange experiments with Pi^{32} or ADP^{32} would not be capable of yielding further information about possible reaction mechanisms.

In an attempt to account for the ADP and phosphate requirements of the transfer reaction, Levintow and Meister (14) have suggested that the transfer reaction consists of a complete reversal of the synthetic reaction followed by the synthetic reaction. Consideration of the following evidence renders this theory unlikely. The extremely low concentration of ATP formed as a result of the reverse reaction would permit the forward reaction to proceed at a rate slow in comparison to the transfer rate actually observed. One could conclude from their theory that the several-fold greater effectiveness of arsenate as a cofactor as compared to phosphate, in the transfer reaction is the result of the formation of the ADP-arsenate analog of ATP and that this ADP-arsenate compound is a more effective reagent in the synthesis reaction than ATP. Data already presented indicate that either ADP-arsenate is not formed, or, if formed, has a very short half-life. In either event the data do not permit the conclusion that ADP-arsenate is an intermediate in the glutamine transfer reaction. Furthermore, the proposal that the transfer reaction occurs by a reversal of glutamine synthesis followed by a resynthesis of glutamine, is inconsistent with the facts that manganous ions are more effective in the transfer reaction while magnesium ions are more effective in the synthetic reaction. Finally, if this mechanism were the correct one, there should be an exchange of radioactive glutamate into glutamine during the transfer reaction, the rate of this exchange should equal the rate of the transfer reaction and it should be increased by the addition of arsenate. That such is not the case has been shown in table V. The rate of exchange is about 2 % of the rate of transfer and this rate of exchange is decreased by the addition of arsenate in the place of phosphate, whereas the rate of the transfer reaction is increased by arsenate.

The data presented in this paper suggest the following conclusions concerning the mechanisms of the reactions catalyzed by the glutamine synthesizing enzyme: 1) All intermediates are enzyme-bound. 2) The formation of a simple enzyme-phosphate, enzyme-pyrophosphate, enzyme-ADP, or enzyme adenylate complex can no longer be considered as likely first steps in the synthetic reaction. More complicated intermediates must, therefore, be sought.

There is as yet no direct unequivocal evidence for

such an intermediate. All of the published data are, however, consistent with the hypothesis that glutamate, ATP and enzyme interact to form a glutamate-ATP-enzyme complex which can then react reversibly with ammonia to form glutamine, ADP and Pi. The formation of this complex from the glutamate side would appear to be more rapid with magnesium ions as compared to manganous ions, while its formation from the glutamine side is more rapid in the presence of manganous ions. It would seem that if the complex exists, it must be quite stable since the exchange of P_i^{32} and ADP^{32} into ADP is very slow in the absence of ammonia. The corresponding arsenate complex is evidently more reactive, thus causing a more rapid transfer reaction as well as a more rapid hydrolysis of glutamine.

It is interesting to compare and contrast these suggestions concerning the mechanism of the synthesis of the amide bond of glutamine with the recently proposed mechanisms for the ATP-dependent syntheses of certain peptide bonds (17, 18, 20, 28, 29).

The evidence presented by these workers suggests phosphorylation of the enzyme as an initial step in the enzymatic formation of these particular peptide bonds. However, since extremely low concentrations of ammonia are so effective in causing exchange of Pi into ATP in the glutamate synthesizing system, perhaps a re-examination of these other systems, using carefully purified enzymes, would be worthwhile. It appears possible, however, that the reaction mechanism for the formation of the amide bond of glutamine may be fundamentally different from those reaction mechanisms presently thought to be involved in peptide bond synthesis.

Summary

An improved method has been described for obtaining from dried green peas in highly purified form the enzyme catalyzing the synthesis of glutamine from glutamate and ammonia. The equilibrium constant for the overall reaction has been determined and the enzyme has been characterized with respect to its affinities for the reactants. The glutamyl transfer reactions of this enzyme have been further characterized. It is concluded that these transfer reactions and the arsenate-activated hydrolysis of glutamine are manifestations of the overall synthetic activity of the enzyme. The mechanism of the synthesis reaction has been studied by means of the enzyme-catalyzed exchange of radioactive orthophosphate and ADP into ATP. The existence of a glutamate-ATP-enzyme complex as an intermediate is indicated and it is suggested that this intermediate is common to the glutamine synthesis reaction and the glutamyl transfer reactions.

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SUCCINOXIDASE AND CYTOCHROME OXIDASE IN BARLEY ROOTS[1,2]

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It has been suggested that the terminal oxidase concerned with the normal respiration in most plants is cytochrome oxidase (7, 8). Evidence from reconstructed systems suggests that other enzymes can also operate as terminal oxidase systems (2, 15, 21, 23, 25, 28, 30). Although tyrosinase and ascorbic oxidase may function there is no experimental evidence that they make energy available for endergonic processes. There is evidence to associate salt accumulation and salt respiration with the cytochrome system (19, 29). If no cytochrome oxidase were present in tissues which accumulate salt some revision of the Lundegårdh hypothesis (20, 24) of salt accumulation would be required. Consequently, it is especially interesting that James presents evidence that ascorbic oxidase is a terminal oxidase of barley root tips (10, 12) and implies that this oxidase is concerned in salt accumulation (11). James has been unable to extract cytochrome oxidase or detect cytochrome absorption bands in barley roots more than 5 to 6 days old.

The direct demonstration of cytochrome oxidase in older wheat roots has also failed (4, 28), although the characteristic absorption bands of the cytochromes have been observed in the intact roots (20); thus suitable procedures for extraction and assay may be difficult to find.

In barley as in wheat roots the negative results (10, 12) might mean only that cytochrome oxidase is difficult to extract. Since it has been generally accepted that oxidation of succinate proceeds through the classical cytochrome system (8), the demonstration of succinate oxidation and formation in barley roots (16, 17) suggests that succinoxidase and, thus, cytochrome oxidase function. Therefore, an attempt was made to isolate cytochrome oxidase and, as a confirmatory test, the succinoxidase system.

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METHODS AND MATERIALS

PREPARATION OF BARLEY: Cape barley (*Hordeum vulgare*) was used as the source of roots. The grains were soaked in distilled water for 3 to 7 hours at 25° C with 2 or 3 changes of water. In some experiments, an initial soak of one hour in 0.6 % H_2O_2 was used to reduce fungal contamination. The soaked grains were allowed to germinate on wet filter paper in a saturated atmosphere and the seedlings were held thus at 25° C for 2 days. For experiments in which longer growth periods were required, seedlings were transferred after 3 days to waxed boards, with holes through which the roots grew in aerated half-saturated $CaSO_4$ solutions for periods up to 7 days in a room at 25° C. After this treatment the roots were profusely covered with root hairs and often had a curled appearance. The maximum amount of root material that could be grown conveniently for an experiment was about 10 grams.

PREPARATION OF THE ENZYME EXTRACT: The root system of the 2-day-old seedlings was excised at the seed and that of 10-day-old seedlings at about 5 cm from the seed. In some experiments the apical cm was removed but no differences between the root segments were found. After being thoroughly rinsed with distilled water the roots were chilled to 5 to 7° C. The following operations were carried out in a cold room at 0 to 5° C. The roots were washed in the homogenizing medium at 2° C and cut into about 0.5-cm segments. The segments were blotted to remove excess fluid and homogenized in fresh medium, 1.5 gm of roots to 5 ml of medium. Maceration was carried out by hand with a conical Pyrex glass homogenizer, consisting of an outer tube which was immersed in a water-ice bath and a hollow pestle which contained a water-ice mixture. Cooling was important since homogenization of roots required a vigorous action. Cell debris from the unstrained homogenate was removed by centrifugation for 10 minutes at $1000 \times g$. The resulting supernatant was centrifuged at about $10{,}000 \times g$ for 25 to 30 minutes at -2 to 0° C