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## Enzymatic Synthesis of $\beta$ -Aminoglutaramic Acid ( $\beta$ -Glutamine) by Glutamine Synthetase: Evidence for the Utilization of $\beta$ -Aminoglutarylphosphate\*

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Glutamine synthetase from sheep brain catalyzes the synthesis of  $\beta$ -aminoglutaramic acid ( $\beta$ -glutamine) from  $\beta$ -aminoglutarate ( $\beta$ -glutamate) and ammonia; when hydroxylamine is substituted for ammonia, the monohydroxamate of  $\beta$ -glutamate is formed. When the enzyme was incubated with adenosine diphosphate and chemically synthesized  $\beta$ -aminoglutarylphosphate, adenosine triphosphate was formed. The data are consistent with previous findings that indicate intermediate formation of enzyme-bound  $\gamma$ -carboxyl-activated glutamic acid in glutamine synthesis, and they support the hypothesis that enzyme-bound glutamylphosphate is formed in the reactions catalyzed by glutamine synthetase.

Recent investigations in this laboratory on the mechanism of the enzymatic synthesis of glutamine by glutamine synthetase led to the conclusion that this reaction involves enzyme-bound  $\gamma$ -carboxyl-activated glutamic acid; the available data are consistent with the intermediate formation of  $\gamma$ -glutamylphosphate (Krishnaswamy *et al.*, 1960, 1962; Meister *et al.*, 1962). However, studies with chemically synthesized  $\gamma$ -glutamylphosphate and attempts to isolate the presumed intermediate from the enzyme have thus far been hindered by the extreme lability of  $\gamma$ -glutamylphosphate and its considerable tendency to cyclize to yield pyrrolidone carboxylate. Both D-glutamate and L-glutamate are substrates for several of the reactions catalyzed by glutamine synthetase (Levintow and Meister, 1953; Meister, 1962), and it is therefore evident that the amino group of the substrate need not be in a specific position for enzymatic activity. The present studies were carried out in an attempt to learn whether the enzyme would act on a substrate in which the amino group was attached to the third possible position of the glutamate carbon chain, i.e.,  $\beta$ -glutamic acid.<sup>1</sup> The answer to this question is of special interest

because the  $\beta$ -aminoglutarylphosphate intermediate that might be postulated in the enzymatic synthesis of  $\beta$ -glutamine would be expected to be relatively stable as compared to  $\gamma$ -glutamylphosphate, and might therefore be useful in experiments on the mechanism of this reaction.

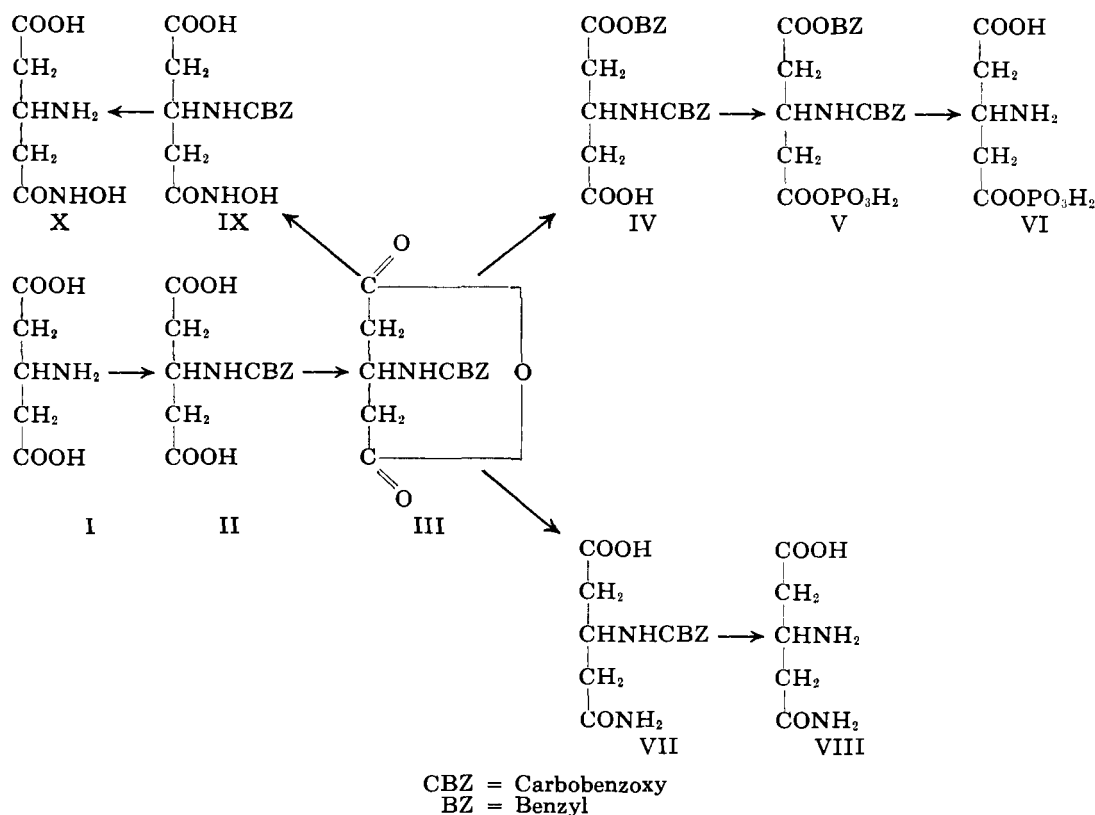
The present communication describes the enzymatic synthesis of  $\beta$ -glutamine and the corresponding hydroxamate from  $\beta$ -glutamate. We have also found that glutamine synthetase catalyzes the synthesis of adenosine triphosphate from adenosine diphosphate and chemically synthesized  $\beta$ -aminoglutarylphosphate.

### EXPERIMENTAL

**Materials.**—Glutamine synthetase was isolated from sheep brain as described previously (Pamijans *et al.*, 1962). Adenosine [ $8\text{-}^{14}\text{C}$ ]diphosphate was purchased from Schwarz BioResearch, Inc., Orangeburg, N. Y. Inorganic [ $^{32}\text{P}$ ] phosphate was obtained from Atomic Energy of Canada, Ltd., Ottawa. Sodium phosphoenolpyruvate was a product of the Sigma Chemical Co., St. Louis, Mo., and crystalline pyruvate kinase was obtained from California Corp. for Biochemical Research. Diethyl glutaconate was purchased from Nutritional Biochemical Corp., Cleveland, Ohio. The other compounds used in these studies were obtained as previously described (Pamijans *et al.*, 1962; Krishnaswamy *et al.*, 1962).

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<sup>1</sup> In this paper, the trivial terms  $\beta$ -glutamic acid and  $\beta$ -glutamine are used for  $\beta$ -aminoglutaric acid and  $\beta$ -aminoglutaramic acid, respectively.



$\beta$ -Glutamine (VIII).— $\beta$ -Glutamic acid (I) was prepared by amination of diethyl glutaconate as described by Feuer and Swartz (1955), and converted to the *N*-carbobenzoxy derivative (II) by the general procedure of Bergmann and Zervas (1932). The product was obtained in 80% yield; mp (Fisher-Johns Block) 163–165° (uncorr).

*Anal.* Calcd. for  $C_{13}H_{15}O_6N$ : C, 55.5; H, 5.33; N, 4.98. Found: C, 55.6; H, 5.41; N, 5.14.

The carbobenzoxy derivative was converted to the anhydride (III) as follows: 1.5 g of the carbobenzoxy-amino acid was dissolved in 10 ml of acetic anhydride and heated on a steam bath for 30 minutes. The solution was cooled and the excess acetic anhydride was removed by flash evaporation; the residual oil was treated with 15 ml of a mixture (1:1, v/v) of acetone and diethyl ether. Ammonia was bubbled into the solution at 0° for 15 minutes. A white precipitate, which formed immediately, was filtered off, and when ammonia was bubbled into the filtrate no further precipitation occurred. The product was washed with acetone and dissolved in the minimal amount of water. On acidification of this solution with hydrochloric acid, crystalline *N*-carbobenzoxy- $\beta$ -glutamine (VII) separated. The product was recrystallized from hot water; yield, 0.9 g (60%); mp 184–185° (uncorr).

*Anal.* Calcd. for  $C_{13}H_{15}O_5N_2$ : C, 55.7; H, 5.72; N, 10.0. Found: C, 55.8; H, 6.01; N, 10.0.

$\beta$ -Glutamine (VIII) was obtained by catalytic hydrogenolysis of the carbobenzoxy derivative (Bergmann and Zervas, 1932), and was crystallized successively from hot water and from water-acetone; mp 245° (decomp).

*Anal.* Calcd. for  $C_5H_9O_3N_2$ : C, 41.1; H, 6.85; N, 19.2. Found: C, 40.7; H, 7.04; N, 18.7.

$\beta$ -Aminoglutarylphosphate (VI).—*N*-Carbobenzoxy- $\beta$ -glutamic acid anhydride (III) (0.7 g) was heated with 20 ml of benzyl alcohol at 100° for 6 hours. Excess benzyl alcohol was removed by flash evaporation and the residue was dissolved in 20 ml of chloroform. The chloroform solution was extracted with several por-

tions of aqueous 5% sodium bicarbonate, and the combined bicarbonate solution (60 ml) was acidified by addition of 6 *N* hydrochloric acid. A crystalline precipitate of *N*-carbobenzoxy- $\beta$ -glutamic acid monobenzyl ester (IV) was obtained; yield, 0.5 g (50%); mp 98–99° (uncorr).

*Anal.* Calcd. for  $C_{20}H_{21}O_6N$ : C, 64.7; H, 5.66; N, 3.77. Found: C, 64.6; H, 5.61; N, 4.12.

This product was converted to the corresponding acylphosphate (V) by a procedure analogous to that used previously for the preparation of *N*-carbobenzoxy- $\alpha$ -benzyl- $\gamma$ -L-glutamyl-L- $\alpha$ -aminobutyrylphosphate (Nishimura *et al.*, 1964; see also Meister and Scott, 1961). *N*-Carbobenzoxy- $\beta$ -glutamic acid monobenzyl ester (IV) (371 mg; 1 mmole) was dissolved in 5 ml of 75% aqueous pyridine. Phosphoric acid (1 mmole) and *N,N'*-dicyclohexylcarbodiimide (4 g) dissolved in 5 ml of pyridine at 0° were added, and the reaction mixture was stirred at 0°. After 1 hour an additional 1 mmole of phosphoric acid in 1 ml of 75% aqueous pyridine was added, and the mixture was stirred for another hour. The mixture was filtered by suction and the residue on the filter was washed with 2 ml of 75% pyridine. The filtrate separated into two layers. The lower layer, which contained virtually all of the product, was mixed with 50 ml of cold petroleum ether. This solution also separated into two layers, and the lower layer of this mixture was mixed with 5 ml of cold 2 *M* lithium chloride. After addition of 300 ml of cold acetone, a voluminous white precipitate formed. The dried product, which weighed 350 mg, represented a yield of about 30% of the desired mixed anhydride as judged by the hydroxamic acid reaction. The product was converted to the free aminoacyl phosphate (VI) by catalytic hydrogenolysis as previously described (Meister and Scott, 1961; Nishimura *et al.*, 1964). It was stable for at least 1 hour in aqueous solution at 26°, but was destroyed rapidly on heating for 1 minute at 100°. Paper electrophoresis in 6.7% formic acid (*vide infra*) of such solutions after hydrogenolysis revealed a compound which

TABLE I  
PAPER CHROMATOGRAPHY AND ELECTROPHORESIS OF  $\beta$ -GLUTAMIC ACID,  $\beta$ -GLUTAMINE,  
AND  $\beta$ -GLUTAMIC ACID MONOHYDROXAMATE

Compound	$R_F$ Values in Solvent <sup>a</sup>				Electrophoresis <sup>b</sup>	
	a	b	c	d	e (cm)	f (cm)
$\beta$ -Glutamic acid	0.48	0.23	0.20–0.24 (streak)	0.27	–95	+8
$\beta$ -Glutamine	0.33	0.64	0.17	0.21	–101	–0.5
$\beta$ -Glutamic acid mono- hydroxamate	0.48	0.22	0.24	0.30	–82	
Glutamic acid	0.46	0.17	0.20	0.28	–74	
Glutamine	0.31	0.56	0.12	0.25	–76	
$\gamma$ -Glutamylhydroxamic acid	0.27		0.10	0.30		

<sup>a</sup> Ascending chromatography on Whatman No. 1 paper was carried out in the following solvents: (a) formic acid–tertiary butanol–water (15:70:15); (b) phenol–water– $\text{NH}_4\text{OH}$  (conc.) (80:20:1); (c) butanol–acetic acid–water (4:1:1); (d) ethanol–water (77:23). <sup>b</sup> Electrophoresis on Whatman 3 MM paper was carried out as follows: (e) 6.7% formic acid; 157-cm strips, 105 minutes, 57 v/cm; temperature, 46° (Savant apparatus). (f) Sodium acetate buffer (0.05 M, pH 5.5); 30-cm strips; 240 minutes; 10 v/cm; initial temperature, 26° (Spinco apparatus). Distances in direction of anode are given in cm from point of application.

was ninhydrin-positive and contained phosphate; semiquantitative estimation of  $\beta$ -glutamate (based on color formation on the paper strip with ninhydrin) and determination of inorganic phosphate (Fiske and SubbaRow, 1925) after elution of the compound with water gave, within experimental error, equivalent values. Treatment of solutions of the product with hydroxylamine gave an amino acid hydroxamate which was indistinguishable from the chemically synthesized monohydroxamate of  $\beta$ -glutamic acid (X) on paper electrophoresis (*vide infra*).

**$\beta$ -Aspartylphosphate and  $\beta$ -Alanylphosphate.**— $\beta$ -Aspartylphosphate was prepared via *N*-carbobenzoxy aspartic acid— $\alpha$ -benzyl ester (Bergmann *et al.*, 1933) by the general procedure described above. This compound was first prepared by Black and Wright (1955) by reaction of the corresponding acyl chloride with silver phosphate.  $\beta$ -Alanylphosphate was prepared via carbobenzoxy- $\beta$ -alanine by the general procedure described; a preparation obtained in a similar manner was used earlier in studies on carnosine biosynthesis (Kalyankar and Meister, 1959).

**Monohydroxamate of  $\beta$ -Glutamic Acid (X).**—*N*-Carbobenzoxy- $\beta$ -glutamic acid (200 mg) was converted to the anhydride as described above. The anhydride was treated with a solution of hydroxylamine (prepared by mixing 3 ml of 2 M  $\text{NH}_4\text{OH}\cdot\text{HCl}$  and 3 ml of 1.8 M NaOH) and allowed to stand for 1 hour at 26°. After catalytic hydrogenation with palladium (Bergmann and Zervas, 1932), the volume of the solution was reduced to about 5 ml in a flash evaporator and then applied to the top of a Dowex 50 ( $\text{H}^+$ ; 1  $\times$  8 cm) column, which was washed with water. The amino acid hydroxamate was then eluted with 2.5 N  $\text{NH}_4\text{OH}$ , and the effluent was repeatedly evaporated to dryness under reduced pressure. The residue (100 mg) was crystallized from water–acetone; yield (first crop), 25 mg (22%); mp 240° (decomp).

*Anal.* Calcd. for  $\text{C}_8\text{H}_{10}\text{O}_4\text{N}_2$ : N, 17.3. Found: N, 17.5.

## METHODS

**Enzymatic Activity.**—Enzymatic activity was determined by measurement of phosphate or hydroxamate formation as previously described (Pamijans *et al.*, 1962). The composition of the assay system was the same as that used previously except that the final concentration of  $\text{Mg}^{2+}$  was 0.02 M rather than 0.05 M; under these conditions the enzymatic activity was 1.3

times that observed with the higher concentration of  $\text{Mg}^{2+}$ .

**Paper Chromatography and Electrophoresis.**<sup>2</sup>—Paper chromatography and electrophoresis of the  $\beta$ -amino acids was carried out as described in Table I. After treatment of the dried papers with ninhydrin (0.25% in acetone) followed by heating at 110°, the  $\beta$ -amino acids gave a brown color. Unlike most  $\alpha$ -amino acids no color was obtained on heating to 60° for 10 minutes or after 24 hours at 26°. With larger amounts of amino acid or prolonged heating, brown-purple spots were obtained.  $\beta$ -Aminoglutarylphosphate was located by treatment of the paper with ninhydrin followed by heating, and by spraying with the phosphate reagent of Hanes and Isherwood (1949) after electrophoresis at 0° (6.7% formic acid, 90-cm strips; 33 v/cm; 90 minutes; Gilson apparatus).  $\beta$ -Glutamate and  $\beta$ -aminoglutarylphosphate moved 12.5 and 2.5 cm, respectively, in the direction of the negative electrode.

**Determination of ATP.**—The reaction mixtures (0.5 ml; see Table III) were treated with 0.05 ml of 18%  $\text{HClO}_4$  and the precipitated protein was removed by centrifugation. The supernatant solutions were adjusted to pH 7 by addition of 1 N KOH, and the precipitated potassium perchlorate was removed by centrifugation. Unlabeled ATP (5  $\mu$ moles) was added, and an aliquot of each solution was applied to the top of a Dowex 1-formate column (1  $\times$  2 cm) which was washed successively with (a) water (2 ml), (b) 0.3 M ammonium formate (pH 5; 20 ml), (c) 0.6 M ammonium formate (pH 6; 20 ml), (d) 0.05 M ammonium formate–4 N formic acid (20 ml), and (e) 0.5 M ammonium formate–4 N formic acid (10 ml). AMP and ADP appeared in fraction (c), ADP appeared in fraction (d), and ATP was eluted exclusively in fraction (e). The radioactivity of the several fractions was determined by plating aliquots on planchets and counting in an automatic gas-flow counter.  $^{32}\text{P}$  was determined in the presence of  $^{14}\text{C}$  by covering the planchet with a section of photographic film. ATP was also separated from AMP and ADP by paper electrophoresis as previously described (Nishimura *et al.*, 1964; Sato *et al.*, 1963); the values obtained in these determinations agreed closely with those obtained by chromatography as described.

<sup>2</sup> The authors are grateful to Dr. Daniel Wellner for assistance and advice in connection with the electrophoretic studies

## RESULTS

**Enzymatic Synthesis of  $\beta$ -Glutamine and the Corresponding Hydroxamic Acid.**—When  $\beta$ -glutamate was incubated with glutamine synthetase in the presence of ATP,  $Mg^{2+}$  ions, and hydroxylamine, substantial hydroxamic acid formation occurred. The reaction proceeded at about 19% of the rate observed with L-glutamate (Table II), and paper electrophoretic

TABLE II  
ENZYMATIC SYNTHESIS OF  $\beta$ -GLUTAMINE AND THE  
CORRESPONDING HYDROXAMIC ACID

Reaction Mixtures <sup>a</sup>	Product ( $\mu$ moles)
$\beta$ -Glutamate + $NH_4^+$	0.11
L-Glutamate + $NH_4^+$	1.52
$\beta$ -Glutamate + $NH_2OH$	0.31
L-Glutamate + $NH_2OH$	1.62

<sup>a</sup> The reaction mixtures consisted of glutamine synthetase (13.3  $\mu$ g; specific activity, 115), imidazole-HCl buffer (pH 7.2; 50  $\mu$ moles), 2-mercaptoethanol (25  $\mu$ moles),  $NH_2OH$  or  $NH_4Cl$  as indicated (100  $\mu$ moles), ATP (10  $\mu$ moles),  $MgCl_2$  (20  $\mu$ moles), and amino acid as indicated (50  $\mu$ moles) in a final volume of 1.0 ml; 37°; 15 minutes. Values are based on hydroxamate formation (with  $NH_2OH$ ) or phosphate formation (with  $NH_4Cl$ ).

studies revealed the formation of a compound which exhibited the properties of authentic  $\beta$ -glutamic acid monohydroxamate. Analogous studies with  $\beta$ -glutamate and ammonia indicated the formation of  $\beta$ -glutamine at about 7% of the rate observed for L-glutamine synthesis. A large-scale enzymatic preparation of  $\beta$ -glutamine in which virtually all of the  $\beta$ -glutamate added was utilized for amide synthesis was carried out by the following procedure in which pyruvate kinase and phosphoenolpyruvate were employed as an ATP-regenerating system.

A mixture consisting of  $\beta$ -glutamate (1.9 mmoles), ATP (0.5 mmole),  $NH_4Cl$  (5 mmoles),  $MgCl_2$  (1 mmole), and sodium phosphoenolpyruvate (2 mmoles) in 30 ml of 0.005 M 2-mercaptoethanol was adjusted to pH 7.2 with 0.1 N NaOH. Glutamine synthetase (specific activity 84; 9 mg; 1 ml) and pyruvate kinase (5 mg in 0.5 ml of saturated  $(NH_4)_2SO_4$ ) were added and the mixture was incubated at 37° for 48 hours. A slight precipitate, which formed during incubation, was removed by centrifugation and discarded, and 45 ml of cold absolute ethanol was added. The precipitated protein was washed with 20 ml of 50% ethanol, and the combined supernatant solution and washing were evaporated to low volume under reduced pressure and applied to the top of a Dowex 50 column ( $H^+$ ;  $2 \times 17$  cm). The column was washed with water until the effluent was no longer acid, and the amino acid was then eluted with 2.5 N  $NH_4OH$ . The effluent containing the product was repeatedly evaporated in a flash evaporator. The dried product weighed 260 mg; it melted at 235–240°, and after one crystallization (acetone-water) it melted at 245°.<sup>3</sup>

Anal. Calcd. for  $C_5H_{10}O_3N_2$ : N, 19.2. Found: N, 18.8.

In contrast to glutamine,  $\beta$ -glutamine gave no color on treatment with Nessler's solution at 26° for 5 minutes. On brief heating of  $\beta$ -glutamine with Nessler's solution a positive reaction was obtained for ammonia. Paper chromatography and electrophoresis revealed a single component which moved with authentic  $\beta$ -gluta-

mine in six different systems (Table I) and gave the characteristic brown color on treatment with ninhydrin. Comparison of the infrared-absorption curves of the chemically synthesized and enzymatically synthesized  $\beta$ -glutamines revealed no significant differences.

**Enzymatic Synthesis of ATP from ADP and  $\beta$ -Aminoglutarylphosphate.**—When the enzyme was incubated with  $\beta$ -aminoglutarylphosphate,  $[8-^{14}C]ADP$ , and  $Mg^{2+}$  ions, significant quantities of ATP were formed (Table III). The synthesis of ATP was markedly reduced when the enzyme,  $Mg^{2+}$  ions, or the mixed anhydride were separately omitted, or when the anhydride was hydrolyzed prior to addition to the reaction mixture by heating at 100° for 1 minute. The formation of some ATP when the anhydride was omitted or hydrolyzed could be due to the presence of a small amount of adenylate kinase in the enzyme preparation, or to reversal of the synthesis reaction (Levintow and Meister, 1954) provided that the reaction mixture was sufficiently contaminated with ammonia. Very little  $[^{32}P]ATP$  was formed when  $[^{32}P]$ phosphate was added to the reaction mixtures (Table III), indicating that only a very small fraction of the ATP formed can be due to the reversal of synthesis. It therefore appears that most of the "blank" ATP formation is due to adenylate kinase; however, the amount of ATP formed in the absence of the anhydride is small compared to the total ATP formed in the complete system. Thus the ATP formation in excess of the "blank" in experiment 1 is 15.1  $\mu$ moles or about 70% of the total ATP formed enzymatically; the corresponding values obtained in experiment 2 are between 76 and 87%. When  $\beta$ -aspartylphosphate or  $\beta$ -alanylphosphate were substituted for  $\beta$ -aminoglutarylphosphate, much less ATP was formed. It is possible that these structurally similar acylphosphates have some affinity for the enzyme; additional work on the specificity of the reaction is needed to evaluate this possibility.

## DISCUSSION

The finding that  $\beta$ -glutamate is a substrate for glutamine synthetase indicates that the position of the amino group on the glutamate carbon chain is not crucial for enzymatic activity.<sup>4</sup> It would be of interest to learn whether the enzyme distinguishes between the carboxyl groups of  $\beta$ -glutamate; studies on the optical isomers of  $\beta$ -glutamine which may be expected to aid in answering this question are in progress. The ability of glutamine synthetase to catalyze the synthesis of both isomers of glutamine from the respective optical isomers of glutamate, as well as the formation of  $\beta$ -glutamine from  $\beta$ -glutamic acid, reflects the remarkably low specificity of this enzyme. The relative rates of synthesis of D- and L-glutamine can be influenced to some extent by variation of the concentration and type of metal ion. It seems possible that divalent metal ions are involved in the binding of amino acids

<sup>4</sup> The rate of the enzymatic synthesis of  $\beta$ -glutamine is of the same order as observed with a number of glutamate analogs that have  $\alpha$ -amino groups. In addition to the glutamate analogs previously reported (Meister, 1962), recent studies in this laboratory have shown that the *threo* and *erythro* isomers of  $\gamma$ -hydroxyglutamate are substrates for sheep brain-glutamine synthetase. (We are indebted to Dr. Milton Winitz and to Dr. Elijah Adams for samples of the  $\gamma$ -hydroxyglutamic acids.) Goldstone and Adams have independently observed the synthesis of the  $\gamma$ -hydroxy-glutamines by purified rat liver glutamine synthetase (personal communication from Dr. Elijah Adams). It is of interest that  $\gamma$ -hydroxyglutamine has recently been isolated from *Phlox decussata* (Brandner and Virtanen, 1963).

<sup>3</sup> The question as to whether the product is a single isomer or a mixture of isomers is under study.

TABLE III  
 ENZYMIC SYNTHESIS OF ATP FROM ADP AND  $\beta$ -AMINOGLUTARYLPHOSPHATE ( $\beta$ -glu  $\sim$  P)

Expt	Reaction Mixtures <sup>a</sup>	ATP Formed $\mu$ moles	Net ATP Formed with $\beta$ -Glu $\sim$ P $\mu$ moles
1	Complete system (enzyme + ADP + $Mg^{2+}$ + $\beta$ -glu $\sim$ P)	23.9	15.1
	Complete system minus $Mg^{2+}$	3.2	
	Complete system minus $\beta$ -glu $\sim$ P	8.8	
	Complete system minus enzyme	2.0	
	Complete system with hydrolyzed $\beta$ -glu $\sim$ P	8.1	
2	Complete system (enzyme + ADP + $Mg^{2+}$ + $\beta$ -glu $\sim$ P)	26.4 (25.4) <sup>b</sup>	21.7 (19.3)
	Complete system (enzyme + ADP + $Mg^{2+}$ + $\beta$ -glu $\sim$ P) <sup>c</sup>	28.0 (31.5)	23.3 (25.4)
	Complete system minus enzyme	1.1 (0.1)	
	Complete system with hydrolyzed $\beta$ -glu $\sim$ P	4.7 (6.1)	
	Complete system with $\beta$ -aspartyl $\sim$ P in place of $\beta$ -glu $\sim$ P	10.1 (9.6)	
	Complete system with $\beta$ -alanyl $\sim$ P in place of $\beta$ -glu $\sim$ P	8.4 (7.9)	

<sup>a</sup> The reaction mixtures contained Tris-HCl buffer (pH 7.3; 50  $\mu$ moles),  $MgCl_2$  (5  $\mu$ moles), [ $8-^{14}C$ ]ADP (0.24  $\mu$ mole, 256,000 cpm in expt 1; 0.175  $\mu$ mole, 450,000 cpm in expt 2), glutamine synthetase (0.4 mg, specific activity, 110 in expt 1; 0.35 mg, specific activity 171 in expt 2), and  $\beta$ -aminoglutarlylphosphate (1  $\mu$ mole) in a final volume of 0.5 ml. The compositions of the individual reaction mixtures are indicated in the table; where present, the anhydrides were added last. Incubation was carried out at 20° for 20 minutes. ATP was determined after separation on Dowex-1 formate as described in the text. <sup>b</sup> Values in parentheses were obtained in analyses carried out on ATP separated by electrophoresis (see text). <sup>c</sup> When carried out in the presence of [ $^{32}P$ ]phosphate (3.5  $\mu$ moles; 10<sup>7</sup> cpm), less than 1  $\mu$ mole of [ $^{32}P$ ]ATP was found.

to the enzyme. However, further studies on the relationships between substrate specificity and divalent metal ions and on the function of the latter are needed.

The present findings indicate that  $\beta$ -aminoglutarlylphosphate is enzymatically utilized for ATP synthesis, and this observation is consistent with the hypothesis that an enzyme-bound glutamylphosphate intermediate is involved in the reactions catalyzed by glutamine synthetase (Krishnaswamy *et al.*, 1962; Meister *et al.*, 1962). The synthesis of ATP observed in the present studies is of the same order as that found in experiments on the formation of ATP from  $\gamma$ -glutamyl- $\alpha$ -aminobutyrylphosphate and ADP catalyzed by tripeptide synthetase (Nishimura *et al.*, 1964). It seems probable that the mechanisms of the reactions catalyzed by tripeptide synthetase, glutamine synthetase, and perhaps other reactions in which ATP is cleaved to ADP and phosphate involve enzyme-bound acylphosphate intermediates. The enzymatic formation of such an acylphosphate has thus far been demonstrated only in the case of tripeptide synthetase (Nishimura *et al.*, 1963). The major difficulty that has attended efforts to demonstrate formation and utilization of  $\gamma$ -glutamylphosphate by glutamine synthetase arises from the instability of this acylphosphate. Earlier studies in which mixtures containing *N*-carbobenzoyl- $\alpha$ -benzyl- $\gamma$ -glutamylphosphate, glutamine synthetase,  $Mg^{2+}$  ions, and ADP were subjected to hydrogenolysis in the presence of palladium gave results that were consistent with enzymatic utilization of  $\gamma$ -glutamylphosphate for ATP synthesis;<sup>5</sup> however, conditions suitable for the reproducible demonstration of ATP formation in such systems have not yet been developed. Attempts to show enzymatic utilization of *N*-acetyl- $\gamma$ -glutamylphosphate (in the presence of acylamino acid *N*-acylase) in similar studies have not yet succeeded. The marked reactivity of  $\gamma$ -glutamylphosphate and its *N*-acetyl derivative with ammonia in the absence of enzyme hindered earlier attempts to show enzymatic utilization of the acyl phosphate for glutamine synthesis (Levintow and Meister, 1956). Although  $\beta$ -aminoglutarlylphosphate may also be expected to exhibit high reactivity with nucleophilic reagents, it is hoped that additional refinements in experimental technique may permit a test of the utiliza-

tion of this compound in the enzymatic synthesis of  $\beta$ -glutamine. Attempts to demonstrate enzymatic formation of  $\beta$ -aminoglutarlylphosphate are also in progress.

The present communication describes the first chemical and enzymatic syntheses of  $\beta$ -glutamine; this amino acid analog and the corresponding analog of glutamic acid may also prove useful in studies on other enzymatic reactions.

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