Huang, W. Y., and Tang, J. (1969), *J. Biol. Chem. 244*, 1085.
Inouye, K., and Fruton, J. S. (1967), *Biochemistry 6*, 1765.
Inouye, K., Voynick, I., Delpierre, G. R., and Fruton, J. S. (1966), *Biochemistry 5*, 2473.
Kitson, T. M., and Knowles, J. R. (1971a), *Biochem. J. 122*, 241.
Kitson, T. M., and Knowles, J. R. (1971b), *Biochem. J. 122*, 249.
Knowles, J. R. (1970), *Phil.. Trans. Roy. Soc. London, Ser. B 257*, 135.
Knowles, J. R., Sharp, H., and Greenwell, P. (1969), *Biochem. J. 113*, 343.
Kozlov, L. V., Ginodman, L. M., and Orekhovich, V. N. (1967), *Biokhimiya 32*, 1011.
Kozlov, L. V., Ginodman, L. M., Zolotarev, B. M., and Orekhovich, V. N. (1962), *Dokl. Akad. Nauk SSSR 146*, 943.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill, p 246.
Lundblad, R. L., and Stein, W. H. (1969), *J. Biol. Chem. 244*, 154.
Mills, J. N., and Tang, J. (1967), *J. Biol. Chem. 242*, 3093.
Neumann, H., Levin, Y., Berger, A., and Katchalski, E. (1959), *Biochem. J. 73*, 33.
Rajagopalan, T. G., Moore, S., and Stein, W. H. (1966), *J. Biol. Chem. 241*, 4940.
Reid, T. W., and Fahrney, D. (1967), *J. Amer. Chem. Soc. 89*, 3941.
Richmond, V., Tang, J., Wolf, S., Trucco, R., and Caputto, R. (1958), *Biochim. Biophys. Acta 29*, 453.
Stein, T. P., and Fahrney, D. (1968), *Chem. Commun.*, 555.
Tang, J. (1963), *Nature (London) 199*, 1094.
Tang, J. (1971), *J. Biol. Chem. 246*, 4510.

Specific Synthesis of 1-(5-Glutamyl)-2-methylhydrazine by Glutamine Synthetase†

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ABSTRACT: Incubation of methylhydrazine in place of ammonia in the glutamine synthetase system leads to the formation of a specific isomer of 5-glutamylmethylhydrazine, namely, 1-(5-glutamyl)-2-methylhydrazine (I). This was established by the unambiguous synthesis of I and its isomer 1-(5-glutamyl)-1-methylhydrazine (II); these were obtained by reaction of 1-carbobenzoxy-1-methylhydrazine or 1-carbobenzoxy-2-methylhydrazine, respectively, with the mixed anhydride obtained by treatment of α-*tert*-butyl *N*-carbobenzoxy-L-glutamate with ethyl chloroformate, followed by removal of the blocking groups. In the presence of relatively high concentrations of methylhydrazine the product of the enzyme-catalyzed reaction (I) is converted to II; this conversion is nonenzymatic. The findings indicate that methylhydrazine attaches to the ammonia binding site of glutamine synthetase in a specific manner which makes only the nonalkylated nitrogen atom of methylhydrazine available for reaction with enzyme-bound γ-glutamyl phosphate.

In the reaction catalyzed by glutamine synthetase (reaction 1), ammonia may be replaced by hydroxylamine, hydrazine, methylamine, glycine ethyl ester (Speck, 1949; Elliott, 1951; Levintow and Meister, 1954), methylhydrazine (Willis, 1966), and several other amines; under these conditions, the corresponding 5-glutamyl compounds are formed. Methylhydrazine is of particular interest since it possesses two nucleophilic centers. As a consequence, depending upon the nature of the amine-binding site on the enzyme, one might expect either 1-(5-glutamyl)-1-methylhydrazine (II, Figure 1) or 1-(5-glutamyl)-2-methylhydrazine (I) to be the enzymatic product; indeed there is no reason, *a priori*, to exclude formation of both I and II. Study of the available literature on the nonenzymatic reactions of methylhydrazine is consistent with the possibility that both I and II might be formed by glutamine synthetase. Thus, it has been shown that reaction of methylhydrazine with an anhydride or with an ester gives both possible hydrazine derivatives (Hinman and Fulton, 1958; Pedersen, 1964). Willis (1966) reported that incubation of methylhydrazine in the glutamine synthetase system gave two enzymatic products which he characterized as I and II; these were not distinguished.

$$\text{glutamate} + NH_3 + ATP \overset{M^{2+}}{\rightleftharpoons} \text{glutamine} + ADP + P_i \quad (1)$$

While we have in the present work confirmed the experimental observations of Willis (1966), a further investigation of the interaction of methylhydrazine in the glutamine synthetase system has shown that there is only one enzymatic product and that the second product arises by a nonenzymatic reaction between the enzymatically formed product and methylhydrazine. In the work reported here, we have established the identity of both the enzymatic product (I) and the nonenzymatic product (II).

Experimental Approach

In the studies reported by Willis (1966), sheep brain glutamine synthetase was incubated with [^{14}C]methylhydrazine, glutamate, ATP, imidazole-HCl buffer, 2-mercaptoethanol,

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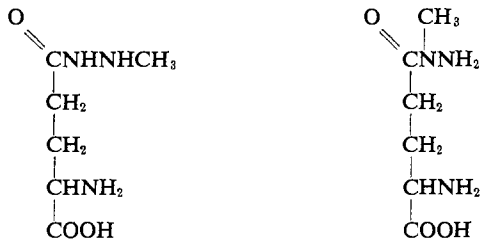

I, 1-(5-glutamyl)-2-methylhydrazine

II, 1-(5-glutamyl)-1-methylhydrazine

FIGURE 1: Structures of the isomeric 5-glutamylmethylhydrazines.

and magnesium chloride; after incubation, the reaction mixture was subjected to descending paper chromatography in a solvent consisting of 1-butanol, acetic acid, and water. Evidence was obtained for the formation of two new compounds (not found in a control experiment with boiled enzyme), which were considered to be the two isomers of 5-glutamylmethylhydrazine. We have repeated the experiment reported by Willis and have obtained substantially the same results. However, when an experiment was carried out under identical conditions except that a concentration of methylhydrazine of 10 mM was used (rather than 32 mM as employed by Willis), only one product was formed (Figure 2). This product appears to be the same as that designated 3C by Willis. The compound designated 4C by Willis was not formed after incubation with the enzyme for 30 min, nor was it formed when the reaction mixture was treated with trichloroacetic acid and then incubated for an additional 57 min. We then carried out a series of experiments in which the concentration of methylhydrazine was varied and it was found that both compounds described by Willis were formed when concentrations of methylhydrazine higher than 10 mM were employed. As indicated in Figure 3, the ratio of the amounts of IE and IIE (corresponding respectively to Willis' compounds 3C and 4C) formed with a constant amount of enzyme decreased considerably as the concentration of methylhydrazine was increased. Thus, with the amount of enzyme used in this experiment and with 20 mM methylhydrazine, about equal amounts of the two compounds were formed; larger amounts of IIE relative to IE were formed with higher concentrations of methylhydrazine. The relative amounts of the two products were also found to depend on the amount of enzyme present in the reaction mixture. Thus, under the conditions described in Figure 3

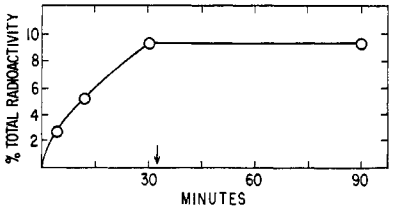

FIGURE 2: Formation of IE catalyzed by glutamine synthetase. The reaction mixtures contained enzyme (9 units), uniformly labeled L-[^{14}C]glutamate (15 μmoles; 250,000 cpm), ATP (10 μmoles), $MgCl_2$ (20 μmoles), methylhydrazine (10 μmoles), 2-mercaptoethanol (25 μmoles), and imidazole-HCl buffer (50 μmoles; pH 7.2) in a final volume of 1.0 ml. After incubation at 37° for 33 min, 1.0 ml of 20% trichloroacetic acid was added to stop the enzyme reaction, and incubation was continued for an additional 57 min. Samples were removed for analysis at the intervals indicated; only IE was found; no IIE could be detected.

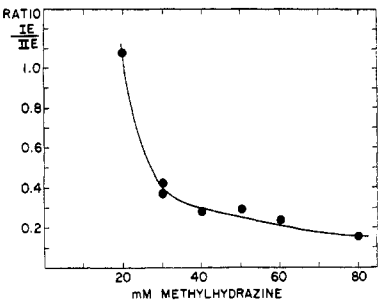

FIGURE 3: Effect of methylhydrazine concentration on the formation of IE and IIE. The reaction mixtures contained enzyme (1.4 units), L-[^{14}C]glutamate (15 μmoles; 250,000 cpm), ATP (10 μmoles), $MgCl_2$ (20 μmoles), methylhydrazine (as indicated), 2-mercaptoethanol (25 μmoles), and imidazole-HCl buffer (50 μmoles; pH 7.2), in a final volume of 1.0 ml. After incubation at 37° for 60 min, 1.0 ml of 20% trichloroacetic acid was added. Samples were removed for determinations of the amounts of IE and IIE formed.

but with a larger amount of enzyme, higher ratios of IE to IIE were obtained.

The experiment described in Table I provides evidence that IIE is formed nonenzymatically from I in the presence of relatively high concentrations of methylhydrazine. In expt 1, in which the concentration of methylhydrazine was 40 mM, both IE and IIE were found (expt 1a). After inactivation of the enzyme by addition of trichloroacetic acid and additional incubation (expt 1b), the amounts of IE and IIE, respectively, decreased and increased, without significant change of the sum of IE plus IIE. In expt 2, in which the

TABLE I: Evidence for Nonenzymatic Conversion of Compound IE to Compound IIE.

Expt	Reaction Conditions[a]	IE (μmoles)	IIE (μmoles)	Sum (IE + IIE) (μmoles)
1a	Initial reaction (A)	1.94	0.86	2.8
1b	(A) + CCl_3COOH	1.47	1.13	2.6
2a	Initial reaction (B)	2.28	0	2.3
2b	(B) + CCl_3COOH	2.36	0.03	2.4
2c	(B) + CCl_3COOH + CH_3NHNH_2	0.53	1.87	2.4

[a] Expt 1: The reaction mixture contained enzyme (7.8 units), L-[^{14}C]glutamate (15 μmoles; 250,000 cpm), ATP (10 μmoles), $MgCl_2$ (20 μmoles), methylhydrazine (40 μmoles), 2-mercaptoethanol (25 μmoles), and imidazole-HCl buffer (50 μmoles; pH 7.2) in a final volume of 1.0 ml. After incubation at 37° for 30 min, aliquots were removed for the determination of IE and IIE (expt 1a) and 1 ml of 20% trichloroacetic acid was added; after incubation at 37° for an additional 30 min, analyses for IE and IIE were done (expt 1b). Expt 2: The reaction mixtures were the same as in expt 1 except that 10 μmoles of methylhydrazine and 9.6 units of enzyme were used. Analysis after 30-min incubation at 37° revealed only IE (expt 2a). In expt 2b, in which trichloroacetic acid was added, and in expt 2c, in which trichloroacetic acid and methylhydrazine (final concentration, 50 mM) were added (in this order), incubation at 37° was also carried out for an additional 30 min.

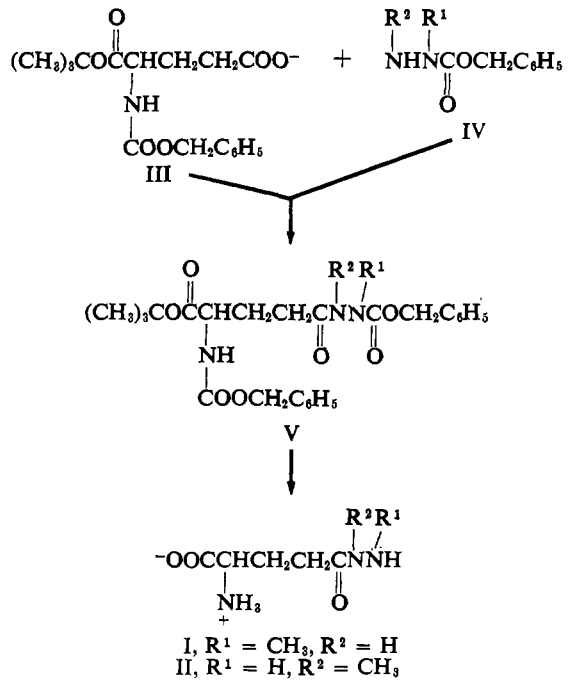

FIGURE 4: Synthesis of the isomeric 5-glutamylmethylhydrazines (see the text).

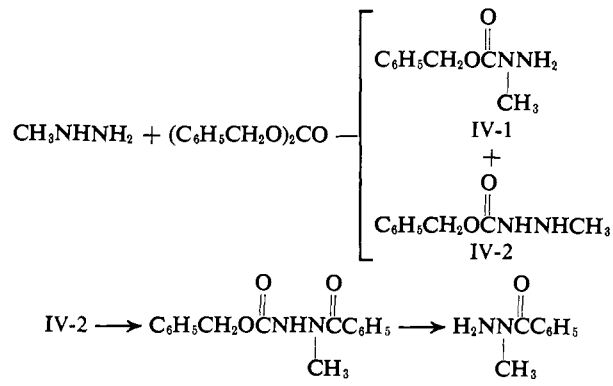

FIGURE 5: Synthesis and characterization of the isomers of 1-carbobenzoxymethylhydrazine (see the text).

initial concentration of methylhydrazine was only 10 mM, no IIE was found initially (expt 2a), nor was an appreciable amount of IIE found after inactivation of the enzyme and further incubation (expt 2b). However, further incubation in the presence of added methylhydrazine (expt 2c) led to a substantial increase in IIE with concomitant decrease in IE. The findings therefore indicate that glutamine synthetase specifically catalyzes the synthesis of IE and that this is nonenzymatically converted to IIE in the presence of relatively high concentrations of methylhydrazine.

To characterize the enzymatic (IE) and nonenzymatic (IIE) reaction products, an unambiguous synthesis of the two 5-glutamylmethylhydrazines was needed. Willis prepared a mixture of these compounds by reaction of 2-pyrrolidone-5-carboxylic acid with methylhydrazine by the method of Gigliotti and Levenberg (1964); it was not possible to distinguish the two isomers. In the present work, I and II (Figure 1) were synthesized as indicated in Figure 4. Compound III was prepared by converting L-glutamate to the corresponding *N*-carbobenzoxy-γ-methyl ester derivative (Hanby *et al.*, 1950); treatment of this compound in *tert*-butyl acetate in the presence of a catalytic amount of 70% perchloric acid gave α-*tert*-butyl γ-methyl-*N*-carbobenzoxyglutamate, which was hydrolyzed to III by treatment with an equivalent amount of sodium hydroxide in aqueous ethanol (Taschner *et al.*, 1961). Compounds IV-1 and IV-2 (Figure 5) were obtained as described by Pedersen (1964). Thus, dibenzyl carbonate (Overberger *et al.*, 1955) and methylhydrazine were refluxed in ethanol to give a mixture of 1-carbobenzoxy-1-methylhydrazide (IV-1) and 1-carbobenzoxy-2-methylhydrazide (IV-2); these were separated by fractional crystallization and extraction. The characterization of IV-1 and IV-2 carried out by Pedersen (1964) was verified in the present work. This characterization was not hindered by the fact that IV-1 contained about 15% of its isomer, IV-2. The most distinctive characteristic of IV-1 and IV-2 was the chemical shift of their corresponding *N*-methyl groups in the nuclear magnetic resonance (nmr); the *N*-methyl group of IV-2 occurred at δ 2.63, while the *N*-methyl group of IV-1 was δ 3.09 as expected for an amide *N*-methyl group. The benzoyl derivative of compound IV-2 was prepared and this compound showed the expected shift of the *N*-methyl resonance to δ 3.01 as a result of the acylation. This benzoyl derivative was also useful as a model compound for testing the required deprotection of the respective isomers of V to give ultimately the desired I and II. As expected from the work of Hofmann *et al.* (1952, 1965), the benzoyl derivative of IV-2 could be converted by treatment with hydrogen and palladium catalyst nearly quantitatively to 1-benzoyl-1-methylhydrazide; this compound was characterized spectrally and by conversion to the hydrazone of *p*-nitrobenzaldehyde (Hinman and Fulton, 1958).

Compound III (Figure 4) was converted to the mixed anhydride with ethyl chloroformate at 0°. In separate syntheses the mixed anhydride was reacted with IV-1 and IV-2 to give the corresponding isomeric protected 5-glutamyl derivatives (V), which were purified by column chromatography on silica gel. The isomers of V were characterized spectrally and analytically, and then converted to I and II by removal of the tetiary butyl ester with anhydrous trifluoroacetic acid at 0°, followed by hydrogenolysis in the presence of palladium catalyst. Compounds I and II were characterized analytically, spectrally, and chromatographically.

Paper and thin-layer chromatographic (tlc) studies of the isomeric 5-glutamylmethylhydrazines and related compounds are summarized in Table II. Chromatographic comparisons of chemically synthesized 1-(5-glutamyl)-2-methylhydrazine (I) and 1-(5-glutamyl)-1-methylhydrazine (II) with the product synthesized by the enzyme (IE) indicate that 1-(5-glutamyl)-2-methylhydrazine (I) is the enzymatically formed product. When chemically synthesized I was incubated in 50 mM methylhydrazine (pH 7.2) for 60 min, substantial conversion to II was demonstrated by paper chromatography. The chromatographic studies also indicate that the compound formed from the enzymatically synthesized product in the presence of high concentrations of methylhydrazine (IIE) is 1-(5-glutamyl)-1-methylhydrazine (II). Compounds IE and I exhibit identical chromatographic mobility and cochromatograph in systems 1b and 2b; analogous studies show that IIE and II exhibit identical chromatographic behavior. Additional evidence for the identity of the enzymatically formed product (IE) with authentic 1-(5-glutamyl)-2-methylhydrazine (I) was obtained by gas–liquid chromatography of the corresponding trimethylsilyl derivatives. As indicated in Table III, the isomeric 5-glutamylmethylhydrazines and several related

TABLE II: Paper and Thin-Layer Chromatographic Data.

Compound	System[a] 1a	1b	2a	2b
5-Glutamylmethylamine	1.03	3.87		1.18
Glutamine	0.92	1.70		0.69
5-Glutamylhydrazine	0.67	1.10		
Methylhydrazine	0.44	3.00	0.62	1.30
1-(5-Glutamyl)-1-methylhydrazine (IIE, II)	0.59	2.04	0.24	0.25
1-(5-Glutamyl)-2-methylhydrazine (IE, I)	0.59	2.51	0.38	0.55

[a] Mobilities are expressed relative to that (R_G = 1.00) of glutamic acid (R_F values: 0.38 and 0.29 in systems 1a and 2a, respectively; movement: 3.0 and 12.0 cm in systems 1b and 2b, respectively). The details of the thin-layer (1a,2a) and paper (1b,2b) chromatography procedures are given in the text. Compounds I and II moved together in solvent 3.

TABLE III: Gas–Liquid Chromatography of Trimethylsilyl Derivatives.[a]

Compound	Retention Time (min)
Glutamic acid	1.34
Glutamine	1.90
5-Glutamylhydrazine	2.98
1-(5-Glutamyl)-1-methylhydrazine (IIE, II)	4.34
1-(5-Glutamyl)-2-methylhydrazine (IE, I)	3.36

[a] The experimental details are given in the text.

compounds can be distinguished by this procedure. The derivatives of the enzymatically formed product (IE) and of chemically synthesized 1-(5-glutamyl)-2-methylhydrazine (I) exhibited identical retention times; when both IE and I were mixed and injected together into the column an enhanced single symmetrical peak was obtained.

Experimental Section

Enzyme Studies. Glutamine synthetase was isolated from sheep brain as described by Rowe *et al.* (1970). Uniformly labeled L-[^{14}C]glutamate (133 Ci/mole) was obtained from New England Nuclear Corp., and 1-methylhydrazine sulfate was obtained from Eastman.

After incubation, the amounts of IE and IIE formed were determined as follows. Aliquots (20–50 μl) of the reaction mixtures were spotted on paper strips (Whatman No. 3MM) and chromatographed by the descending technique on solvent 2 (see below); under these conditions, glutamate and the 5-glutamylmethylhydrazine isomers are effectively separated (Table I; Willis, 1966). The radioactivity present in the several areas of the paper strip was determined with a Nuclear-Chicago strip counter or by scintillation counting.

The apparent K_m value for methylhydrazine, determined by measurement of inorganic phosphate formation under the standard assay conditions described by Rowe *et al.* (1970), was 80 mM; the K_m value for NH_4Cl was 0.3 mM. The corresponding V_{max} values for methylhydrazine and NH_4Cl were the same. In these studies, inorganic phosphate was precipitated as the calcium salt prior to colorimetric assay.

General Methods. All melting points are uncorrected. Infrared (ir) spectra were obtained with a Perkin-Elmer 257 spectrophotometer. KBr pellets were prepared by mixing 1 mg of the sample with 100 mg of anhydrous KBr and then fusing the salt at 15,000–18,000 psi for 5 min *in vacuo*. Nuclear magnetic resonance spectra were obtained with a Varian A-60 spectrometer. Peak positions are given in parts per million downfield from tetramethylsilane as the internal standard; sodium trimethylsilylpropanesulfonate was used as the internal standard in aqueous solutions. The abbreviations s, d, t, q, and m refer to singlet, doublet, triplet, quartet, and multiplet, respectively. Gas–liquid chromatography was carried out with an F and M Model 400 instrument equipped with a hydrogen flame ionization detector. Elemental analyses were performed by Mr. T. Bella of the Microanalytical Laboratory, Rockefeller University, New York.

Paper and Thin-Layer Chromatography. The solvent systems used in this work were (1) 2-propanol–water (4:1, v/v), (2) 1-butanol–acetic acid–H_2O (4:1:5, v/v, upper phase), and (3) 1-butanol–ethanol–0.5 N NH_4OH (4:1:1, v/v). Chromatography was carried out on (a) Brinkman Polygram SIL-N-HR tlc plates, and (b) Whatman No. 3MM paper. The paper chromatograms were run by the descending method. The chromatograms were developed by spraying with 0.3% ninhydrin in acetone (w/v) containing a trace of pyridine. In some studies the chromatograms were sprayed with acidic 2.5% *p*-dimethylaminobenzaldehyde in ethanol to detect methylhydrazine. The 5-glutamylmethylhydrazines both exhibited an R_F value of 0.09 in system 3a.

Preparation and Gas–Liquid Chromatography of Trimethylsilyl Derivatives. Trimethylsilyl derivatives were prepared from *N,O*-bis(trimethylsilyl)acetamide according to the general method of Klebe *et al.* (1966). The dried chromatographic residue or about 1 mg of amino acid was placed in a tube fitted with a serum cap. Dimethylformamide (25–50 μl) and the trimethylacetamide (25–50μl) were added with a syringe and the mixture was heated at 37° for about 1 hr. The samples (2 μl) were injected into a column (183 cm × 4 mm, glass) of "DXO" (Pisano and Bronzert, 1969). The operating parameters were: column temperature, 263°; detector temperature, 305°; nitrogen carrier gas pressure, 70 psi; hydrogen pressure, 20 psi.

Identification of IE as 1-(5-glutamyl)-2-methylhydrazine (I) was carried out as follows. The reaction mixture (1 ml; Figure 2) was spotted on a strip (25 cm wide) of Whatman No. 3MM paper and developed with solvent 2. The band corresponding to IE was located by developing a small part of the chromatogram with ninhydrin. The remaining portion was eluted with 2-propanol–H_2O (4:1), concentrated *in vacuo*, and converted into the trimethylsilyl derivative by treatment with a mixture of dimethylformamide and *N,O*-bis(trimethylsilyl)acetamide at 37° for 1 hr. In an analogous manner, reference samples of the silylated derivatives of I, II, glutamic acid, glutamine, and 5-glutamylhydrazine were prepared; glutamine required 3 hr for maximum reaction. In all cases, except glutamic acid, the trimethylsilyl derivatives were chromatographed within a few hours after preparation, since conversion to other volatile products was observed on standing

at room temperature for longer periods. Even when the minimum reaction time was used, additional minor peaks were observed in the chromatography of I, glutamine, and 5-glutamylhydrazine.

Sodium α-tert-Butyl N-Carbobenzoxy-L-glutamate (III-Na⁺). γ-Methyl-L-glutamate was prepared from L-glutamic acid as described by Hanby *et al.* (1950) in 41% yield (mp 182–183°; reported, 182°). The product gave a single spot on tlc in solvent 1 (R_F 0.53). This was converted in 63% yield to γ-methyl-*N*-carbobenzoxy-L-glutamate. This compound was used for the preparation of α-*tert*-butyl γ-methyl-*N*-carbobenzoxy-L-glutamate by the method of Taschner *et al.* (1961). Purification of this product to remove polyisobutylenes was accomplished either by trituration with pentane or column chromatography on silica gel using 10% ethyl acetate in benzene; the product was obtained in 44% yield: nmr ($CDCl_3$) spectra (given in δ values), 7.29 (5 H, s), 5.70 (1 H, m), 5.06 (2 H, s), 4.26 (1 H, m), 3.60 (3 H, s), 1.7–2.6 (4 H, m), and 1.42 (9 H, s); tlc in 10% ethyl acetate in benzene (I_2 detection) gave a single spot, R_F 0.70. The α-*tert*-butyl γ-methyl-*N*-carbobenzoxy-L-glutamate thus obtained (5.2 mmoles) was mixed with 6.25 ml of 0.8 N NaOH in 90% ethanol. After adding 12 ml of 80% ethanol, the mixture was allowed to stand for 18 hr. The solution was evaporated *in vacuo* to dryness and 50 ml of ethanol was added to the residue and then evaporated *in vacuo*. This process was repeated twice with ethanol and again with two 50-ml portions of benzene. The residue was triturated with cyclohexane and then filtered to yield 1.48 g (79%) of a white hygroscopic salt: mp 63–67°; nmr (D_2O) spectra 7.35 (5 H, s), 5.08 (2 H, d), 4.08 (1 H, m), 1.8–2.5 (4 H, m), and 1.40 (9 H, s). A sample of the product (III-Na⁺) was converted *via* the corresponding acid to the cyclohexylammonium salt: mp 147–149° (149°; Taschner *et al.*, 1961).

1-(α-tert-Butyl N-Carbobenzoxy-5-glutamyl)-1-methyl-2-carbobenzoxyhydrazine (V; R^1 = H, R^2 = CH_3). Sodium α-*tert*-butyl *N*-carbobenzoxyglutamate (857 mg) was placed in a 25-ml erlenmeyer flask and 10 ml of toluene was added. After cooling in an ice–methanol bath, 134 μl (1 mmole) of triethylamine was added. Ethyl chloroformate (238 μl) was added and after stirring at −10° for 1 hr, 428 mg of 1-carbobenzoxy-2-methylhydrazine was added. After standing for 18 hr, the mixture was transferred to a separatory funnel with the aid of 50 ml of toluene and washed with 50 ml of 5% phosphoric acid and then with water until the washings were neutral. After drying over anhydrous potassium carbonate, the solution was filtered and the filtrate was evaporated *in vacuo* to a viscous residue, which was chromatographed on silica gel using an eluent consisting of 20% ethyl acetate in benzene to yield 450 mg (38%) of V (R^1 = H, R^2 = CH_3): nmr (CCl_4) spectra 7.32 (10 H, m), 6.10 (1 H, m), 5.10 (sH, d), 4.99 (sH, d), 4.02 (1 H, m), 2.98 (3 H, s), 1.8–2.6 (4 H, m), and 1.38 (9 H, s). *Anal.* Calcd for $C_{26}H_{33}N_3O_7$: C, 62.5; H, 6.6; N, 8.4. Found: C, 63.2; H, 6.7; N, 8.2.

1-(α-tert-Butyl N-Carbobenzoxy-5-glutamyl)-2-methyl-2-carbobenzoxyhydrazine (V; R^1 = CH_3, R^2 = H). This compound was obtained by the same method used for the preparation of V (R^1 = H, R^2 = CH_3) in 50% yield by substituting 1-carbobenzoxy-1-methylhydrazine for 1-carbobenzoxy-2-methylhydrazine: nmr (CCl_4) spectra 7.30 (10 H, s), 6.10 (1 H, m), 5.06 (4 H, s), 4.12 (1 H, m), 3.11 (3 H, s), 1.8–2.4 (4 H, m), and 1.38 (9 H, s). *Anal.* Calcd for $C_{26}H_{33}N_3O_7$: C, 62.5; H, 6.6; N, 8.4. Found: C, 61.5; H, 6.6; N, 8.3.

1-Carbobenzoxy-2-methylhydrazine (IV-2). This compound was prepared by the method of Pedersen (1964) by refluxing methylhydrazine with dibenzyl carbonate (Overberger *et al.* (1955)). The product was obtained in 23% yield: mp 81–82° (lit. mp 81–82°); nmr ($CDCl_3$) spectra 7.36 (5 H, s), 5.0–6.0 (2 H, m), 5.14 (2 H, s), and 2.63 (3 H, s).

1-Carbobenzoxy-1-methylhydrazine (IV-1). The isomeric hydrazine was prepared essentially as described by Pedersen (1964); part of the residual hydrazine (IV-2) crystallized from the oily product. Extraction with hexane followed by removal of the solvent gave an 85:15 mixture of IV-1 and IV-2; this was used for the preparation of the corresponding isomer of V: nmr (neat) spectra 7.40 (5 H, s), 5.16 (2 H, s), 4.54 (2 H, s), and 3.09 (3 H, s).

1-Benzoyl-2-carbobenzoxy-1-methylhydrazine. Compound IV-2 (0.198 g) was dissolved in 30 ml of toluene. After adding 2 ml of pyridine and cooling to 0°, 1.2 ml of benzoyl chloride in 30 ml of toluene was added dropwise. After stirring for 3 hr, the solution was extracted successively with two portions of 50 ml each of water, 50 ml of 5% phosphoric acid, two portions of 50 ml each of water, 50 ml of 10% potassium carbonate, and two portions of 50 ml each of water. After drying over anhydrous sodium sulfate, the toluene was removed *in vacuo* to yield 2.55 g (82%) of light brown solid. On distillation (110–120°; 100 μ) white crystals were obtained: mp 73–75°; nmr (CCl_4) spectra 7.08 (10 H, m), 4.86 (2 H, s), and 3.01 (3 H, s), *Anal.* Calcd for $C_{16}H_{16}N_2O_3$: C, 67.6; H, 5.6; N, 9.9. Found: C, 67.9; H, 5.8; N, 9.9.

1-Benzoyl-1-methylhydrazine. A mixture of 1-benzoyl-2-carbobenzoxy-1-methylhydrazine (300 mg), 10% palladium on charcoal (30 mg), methanol (30 ml), and acetic acid (0.3 ml) was subjected to hydrogenation at atmospheric pressure. After cessation of carbon dioxide evolution, the solution was filtered through Celite to yield 140 mg (88%) of product: nmr (CCl_4) spectra 7.29 (5 H, m), 3.9–4.9 (2 H, m), and 3.10 (3 H, s). Addition of an ethanolic solution of *p*-nitrobenzaldehyde gave 240 mg (85%) of the corresponding hydrazone: mp 176–178° (lit. mp (Hinman and Fulton, 1958) 172–173°). *Anal.* Calcd for $C_{15}H_{13}N_3O_3$: C, 63.6; H, 4.6; N, 14.8. Found: C, 62.9; H, 4.9; N, 14.7.

1-(5-Glutamyl)-1-methylhydrazine (II). Compound V (R^1 = H, R^2 = CH_3) (143 mg) was placed in a 100-ml round-bottom flask. After cooling in an ice bath, 5 ml of anhydrous trifluoroacetic acid was added. After 30 min the acid was removed *in vacuo*, and benzene (30 ml) was added; the solvent was then removed by evaporation. Addition of benzene followed by evaporation was repeated and the residue then obtained was dissolved in 40 ml of methanol containing 0.3 ml of acetic acid. After adding 20 mg of 10% palladium on charcoal, the solution was flushed with hydrogen and then stirred under hydrogen for 15 min until cessation of carbon dioxide evolution. The solution was filtered through Celite, concentrated to 5 ml, and then treated with 75 ml of anhydrous ether to precipitate the product. After filtering and drying, 33 mg (65%) of product was obtained. A sample was reprecipitated from ethanol–water (10:1) by adding ether: mp 148–140° dec; ir (KBr) spectra (in cm^{-1}) 1640 (amide C=O), 1578 (carboxylate anion), and broad band at 2500–3600 (similar to ir of an authentic sample of 5-glutamylhydrazine); nmr (D_2O–CF_3COOH) spectra 4.15 (1 H, t), 3.45 (3 H, s), 2.93 (2 H, t), and 2.40 (2 H, m). *Anal.* Calcd for $C_6H_{13}N_3O_3$: C, 41.2; H, 7.4; Found: C, 40.6; H, 7.4. A hydrazone was prepared by dissolving a sample in ethanol–H_2O (10:1) and adding an equivalent amount of *p*-nitrobenzaldehyde in ethanol: mp 178–180° dec. *Anal.* Calcd for $C_{13}H_{16}N_4O_5 \cdot H_2O$: C, 47.8; H, 5.5; N, 17.2; Found: C, 47.3; H, 5.2; N, 17.1.

1-(5-Glutamyl)-2-methylhydrazine (I). The procedure fol-

lowed for the synthesis of II was employed; 150 mg of V ($R^1 = CH_3$, $R^2 = H$) was used and 28 mg (53%) of product was obtained: mp 140–155° dec; ir (KBr) spectra nearly superimposable with the ir spectra of 5-glutamylhydrazine; it showed broad absorption at 2500–3500, 1640 (amide C=O), and 1585 (carboxylate anion); nmr (D_2O–CF_3COOH) spectra 4.15 (1 H, t), 3.04 (3 H, s), 2.1–2.9 (4 H, m). *Anal.* Calcd for $C_6H_{13}N_3O_3$: C, 41.2; H, 7.4; Found: C, 40.4; H, 7.5.

Discussion

These studies indicate that glutamine synthetase catalyzes the synthesis of only one of the two isomeric 5-glutamylmethylhydrazines. The unambiguous synthesis of the isomers of 5-glutamylmethylhydrazine establishes the product synthesized by glutamine synthetase as 1-(5-glutamyl)-2-methylhydrazine. The findings also show that 1-(5-glutamyl)-1-methylhydrazine is formed nonenzymatically from 1-(5-glutamyl)-2-methylhydrazine in the presence of excess methylhydrazine apparently by transhydrazinolysis; the formation of 1-(5-glutamyl)-1-methylhydrazine is favored in mixtures containing 1-(5-glutamyl)-2-methylhydrazine and methylhydrazine. This observation and the fact (Hinman and Fulton, 1958; Pedersen, 1964) that both isomeric acylmethylhydrazines are formed when esters and anhydrides react with methylhydrazine stand in marked contrast to the highly specific formation of 1-(5-glutamyl)-2-methylhydrazine by glutamine synthetase. The ammonia binding site of glutamine synthetase seems to be constructed in such a manner as to make only the nonalkylated nitrogen atom of methylhydrazine available for reaction with enzyme-bound γ-glutamyl phosphate. The present findings on the specificity of methylhydrazine utilization by glutamine synthetase suggest the possibility of mapping the ammonia binding site of the active center. This might be accomplished by carrying out studies with various ammonia and glutamate analogs in a manner similar to that previously employed in studies on the mapping of the glutamate binding site of this enzyme (Gass and Meister, 1970; Meister, 1968). It has already been noted that a number of amines can replace ammonia and studies are currently in progress in our laboratory in an effort to obtain data suitable for such a mapping effort.

References

Elliott, W. H. (1951), *Biochem. J. 49*, 106.

Gass, J. D., and Meister, A. (1970), *Biochemistry 9*, 1380.

Gigliotti, H. J., and Levenberg, B. (1964), *J. Biol. Chem. 239*, 2274.

Hanby, W. E., Waley, S. G., and Watson, J. (1950), *J. Chem. Soc. A*, 3239.

Hinman, R. L., and Fulton, D. (1958), *J. Amer. Chem. Soc. 80*, 1895.

Hofmann, K., Haas, W., Smithers, M. J., Wells, R. D., Wolman, Y., Yanaihara, N., and Zanetti, G. (1965), *J. Amer. Chem. Soc. 87*, 620.

Hofmann, K., Lindenmann, A., Magee, M. Z., and Khan, N. H. (1952), *J. Amer. Chem. Soc. 74*, 470.

Klebe, J. F., Finkbeiner, H., and White, D. M. (1966), *J. Amer. Chem. Soc. 88*, 3390.

Levintow, L., and Meister, A. (1954), *J. Biol. Chem. 209*, 265.

Meister, A. (1968), *Advan. Enzymol. 31*, 183.

Overberger, C. G., Palmer, L. C., Marks, B. S., and Byrd, N. R. (1955), *J. Amer. Chem. Soc. 77*, 4100.

Pedersen, C. T. (1964), *Acta Chem. Scand. 18*, 2199.

Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem. 244*, 5597.

Rowe, W. B., Ronzio, R. A., Wellner, V. P., and Meister, A. (1970), *Methods Enzymol. 17A*, 900.

Speck, J. F. (1949), *J. Biol. Chem. 179*, 1405.

Taschner, E., Wasielewski, Cz., Sokolowska, T., and Biernat, J. F. (1961), *Justus Liebigs Ann. Chem. 646*, 127.

Willis, J. E. (1966), *Biochemistry 5*, 3557.