A third estimation for the virus monomer fraction is given by centrifugation data for the extracted RNA. The fraction of intact RNA in a phenol extract was 65%. The apparent range for the sedimentation coefficients was ± 0.8 svedbergs about the median of 30.1. Using the Mandelkern–Flory relation for a random coil,[32] the probable form for RNA in 0.02 *M* phosphate buffer,[33] the corresponding range in molecular weights is ± 5% of the median. The true weight range should be less since at least half of the boundary spread can be accounted for by diffusion. For five samples of TMV totally degraded by urea, the average fraction of intact RNA was calculated to be 64.9 ± 1.6%. This value, however, depends on the value used for the ratio of RNA to protein optical density in the mixture. The two boundaries, presumably RNA and protein, are well separated (see Fig. 1B), but the protein migrates so slowly that it was necessary to use the trace height for the airspace above the meniscus, to calculate protein and total optical density. This procedure is valid so long as the solution contains no low molecular weight ultraviolet absorbing contaminants. The five values for the optical density ratio agreed well, the average being 1.61 ± 0.05 to one. Absorption spectra given by Fraenkel-Conrat and Williams[34] predict an optical density ratio of 1.34:1 for a wave length of 265 mμ and of 2.41:1 for λ = 254. The ratio of 1.6:1 suggests that 75% of the incident light had λ = 265 which seems reasonable.[30]

B. Calculation of Fraction of Virus Degraded.—Clearly the apparent fraction of degraded virus does not correspond directly to the fraction of virus reacted, since the particles have lost mass. Also, presumably, the apparent degraded virus fraction includes some fragments. Considering first the electron microscope data, both the mass of degraded rods and the total mass must be corrected for the mass of material rendered too small to see. Assuming, for the case of 2 to 5 minutes reaction, the average degraded rod is $^7/_{12}$ of monomer length, the average length lost is $^5/_7$ of the length remaining. Then if the fragment fraction in the size range 210–150 mμ is taken as 9% it follows that

$$L_c - L = \frac{5}{700}(w_o L - 9L_c)$$

$$\text{and} \quad w_c = \frac{12}{7}\,\frac{(w_o L - 9L_c)}{L_c}$$

where L_c and L are calculated and observed total length; w_o is observed wt. % of degraded virus and w_c is calculated wt. % of virus degraded. Also, the wt. % for the remaining classes of rods must be multiplied by L/L_c. Analogous corrections were applied to the data for 1 and 6 minute reaction times and the corrected data is given in bold faced type in Table II. If the wt. % for degraded rods and residual monomer are added the total is 71 ± 3%. Statistical analysis of the wt. % for all classes of fragments show that calculated and observed standard deviations agree and that therefore no detectable decrease of fragments occurs with treatment times up to 6 minutes.

To allow for the mass loss in the case of the ultracentrifugation data, the optical density of the two slowest sedimenting components was added to that of the degraded component. For the slower of the two, presumably free protein, which appears motionless at 27,690 r.p.m. the optical density estimate was based on the trace height of the air space above the meniscus. The calculated percentages of free protein (total protein being taken as total optical density × 1/2.6) are close to expectation. The faster component, which appears to vary erratically in magnitude, is probably free RNA. If virus fragments are impervious to 6 minute urea treatment, the only source of free RNA is the firecracker tails of the degraded rods. As noted earlier survival of exposed RNA strands is likely to be erratic.

The average fraction, so calculated, for the virus degraded in reaction times from 2 to 5 minutes, is 54 ± 4%. This value neglects the scattering contribution to optical density. An estimate of the scattering correction was made assuming the optical density would increase by 40%[34] if totally degraded material were assembled into monomers and that the scattering contribution of degraded virus is $^7/_{12}$ that of monomers.[35] The corrected values for the fraction of virus degraded are given in Table I. The average value is 59 ± 4%. (It should be noted that similar correction for the control virus data leaves the 68% value for the monomer fraction virtually unchanged but reduces the "dimer" fraction, closer to the 4% estimated from electron microscope data.) The apparent sedimentation constants for the upper and lower boundary limits were, on the average, 11 svedbergs above and below the constant for the boundary mid point, indicating a range of particle sizes. Allowing for diffusion, the range in sedimentation constants should be about ± 6 svedbergs.

(32) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953.

(33) U. Z. Littauer and H. Eisenberg, *Biochim. Biophys. Acta*, **32**, 320 (1959).

(34) H. Fraenkel-Conrat and R. C. Williams, *Proc. Nat. Acad. Sci.*, **41**, 690 (1955).

(35) G. Oster, P. M. Doty and B. H. Zimm, THIS JOURNAL, **69**, 1193 (1947).

PITTSBURGH, PENNA.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Convenient Syntheses of L-Isoglutamine and L-Isoasparagine through Derivatives Commonly Useful for Peptide Synthesis

BY CHARLOTTE RESSLER[1]

RECEIVED JULY 28, 1959

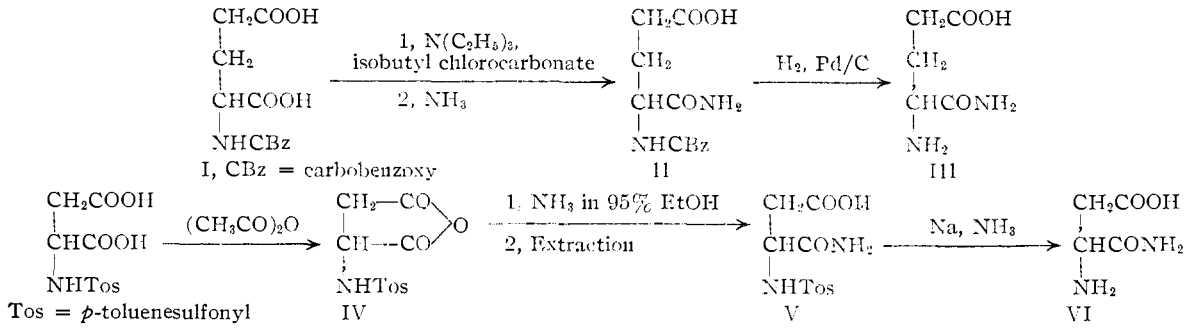
A new route to L-isoglutamine and a simplified route leading to L-isoasparagine are described. Carbobenzoxy-L-glutamic acid was converted through preferential activation of its α-carboxyl group followed by treatment with NH_3 to carbobenzoxy-L-isoglutamine, from which L-isoglutamine was obtained through hydrogenolysis. Direct amidation of *p*-toluenesulfonyl-L-aspartic anhydride yielded *p*-toluenesulfonyl-L-isoasparagine, which, after purification, was converted with sodium in NH_3 to L-isoasparagine. The preparation of *p*-toluenesulfonyl-L-asparagine is also given.

Since glutamine and asparagine occur so widely in nature, both free and combined as constituents of polypeptides and proteins of biological importance, a study of the activity and properties of polypeptides containing the isomeric isoglutamine and isoasparagine residues is of interest. For example, isoglutamine-oxytocin, a synthetic, isoglutamine-containing isomer of oxytocin, has been found to possess the interesting property of inhibiting some of the physiological activities of vasopressin.[2] Synthetic methods have therefore been sought which would provide in reasonable yield and purity sources of L-isoglutamine and L-isoasparagine for such purposes.

Many of the syntheses of isoglutamine and iso-

(1) Institute for Muscle Disease, Inc., New York, N.Y. This work was carried out during the tenure of an Established Investigatorship of the American Heart Association. It was aided by a grant from Chas. Pfizer and Co., Inc.

(2) C. Ressler and J. R. Rachele, *Proc. Soc. Exptl. Biol. Med.*, **98**, 170 (1958).



asparagine that have already been described have been based upon the original syntheses of Bergmann and Zervas,[3] in which the cyclic anhydrides of carbobenzoxy-L-glutamic acid and carbobenzoxy-L-aspartic acid served as the intermediates. These syntheses[4a] suffer from the fact that considerable amounts of the glutamine or asparagine isomer are formed along with the isoglutamine or isoasparagine derivative, and this necessitates careful fractionation of the product which is accompanied by considerable loss.[4b] In other syntheses, protecting groups such as phthaloyl[5,6] or benzoyl[7] have been employed; such derivatives are not highly suitable for use in peptide synthesis because of difficulties associated with removal of the protecting group, or attendant racemization during their incorporation into peptides. Other preparations of isoglutamine[8-12] and isoasparagine[13,14] have involved somewhat circuitous routes because of the presence of the two reactive carboxyl groups in the starting glutamic and aspartic acid derivatives, or yield the racemic product.[15,16]

In this paper a new route to L-isoglutamine and a simplified route leading to L-isoasparagine are presented. These were prepared *via* the carbobenzoxy and *p*-toluensulfonyl (*p*-tosyl) derivatives, respectively, which should, like the carbobenzoxy and *p*-tosyl derivatives of many other amino acids, be useful for peptide synthesis. The economy of steps involved, the low proportion of undesired isomer initially formed, and the ease with which the desired carbobenzoxy-L-isoglutamine and tosyl-L-isoasparagine are isolated and purified are features of the present syntheses, which are illustrated in the accompanying diagram.

The new route to carbobenzoxy-L-isoglutamine was based upon the expectation that the α-carboxyl group of carbobenzoxy-L-glutamic acid would have a considerably greater acid strength than the γ-carboxyl group.[17] It was found that treatment of the triethylamine salt of carbobenzoxy-L-glutamic acid in tetrahydrofuran with only one equivalent of isobutyl chlorocarbonate[18] preferentially activated the α-carboxyl group, and on introduction of NH_3 gas the desired carbobenzoxy-L-isoglutamine precipitated out as a salt. It was of considerable advantage to prepare the mixed anhydride at $-40°$, a temperature somewhat lower than that usually used for such reactions. At $-15°$, some evolution of gas occurred, accompanied possibly by formation of the cyclic anhydride, and the resulting product, which was low-melting, was not so readily purified. That the α-carboxyl group had been activated preferentially at $-40°$ was confirmed by close examination by paper electrophoresis of the crude product after hydrogenolysis, which showed only a minor amount of glutamine. Purified carbobenzoxy-L-isoglutamine(II) was obtained after one or two recrystallizations in a yield of approximately 50%.

Compound II was converted through hydrogenolysis in the usual manner[3] to L-isoglutamine. The homogeneity of the latter was investigated by examination of the melting point and optical rotation, and through the use of paper electrophoresis with barbital buffer at *p*H 8.5. This system was found particularly suitable since it provided an effective separation in several hours between isoglutamine and each of the two substances likely to be present as impurities, glutamine and glutamic acid. No detectable amounts of the latter or of ammonium ion were found in the crystallized product.

The route to *p*-tosyl-L-isoasparagine involves the direct amidation of *p*-tosyl-L-aspartic anhydride. It was found unnecessary to proceed through the benzyl ester intermediate, α-benzyl *p*-tosylaspartate, as in a synthesis of *p*-tosyl-DL-isoasparagine.[14] The conditions employed for the direct amidation of the anhydride were based on those found favorable for the conversion of phthaloylaspartic anhydride to phthaloylisoaspara-

(3) M. Bergmann and L. Zervas, *Ber.*, **65B**, 1192 (1932).

(4) (a) For example, see R. W. Chambers and F. H. Carpenter, THIS JOURNAL, **77**, 1522 (1955); (b) recent exception to this is the amidation of N-trifluoroacetyl-L-aspartic anhydride to the isoasparagine compound in good yield (F. Weygand, P. Klinke and I. Eigen, *Ber.*, **90**, 1896 (1957)).

(5) F. E. King, B. S. Jackson and D. A. A. Kidd, *J. Chem. Soc.*, 243 (1951).

(6) S. W. Tanenbaum, THIS JOURNAL, **75**, 1754 (1953).

(7) A. R. Battersby and J. C. Robinson, *J. Chem. Soc.*, 259 (1955).

(8) (a) C. R. Harington and R. C. G. Moggridge, *ibid.*, 706 (1940); (b) J. M. Swan and V. du Vigneaud, THIS JOURNAL, **76**, 3110 (1954).

(9) R. B. Angier, C. W. Waller, B. L. Hutchings, J. H. Boothe, J. H. Mowat, J. Semb and Y. SubbaRow, *ibid.*, **72**, 74 (1950).

(10) W. J. LeQuesne and G. T. Young, *J. Chem. Soc.*, 1954 (1950).

(11) T. Wieland and H. L. Wiedenmüller, *Ann. Chem.*, **597**, 111 (1955); H. Sachs and H. Waelsch, THIS JOURNAL, **77**, 6600 (1955).

(12) B. A. Borek and H. Waelsch, *J. Biol. Chem.*, **205**, 459 (1953).

(13) W. J. Le Quesne and G. T. Young, *J. Chem. Soc.*, 24 (1952).

(14) M. R. Bovarnick, *J. Biol. Chem.*, **148**, 151 (1943).

(15) Y. Liwschitz and A. Zilkha, THIS JOURNAL, **76**, 3698 (1954).

(16) A. Zilkha and Y. Liwschitz, *J. Chem. Soc.*, 4397 (1957).

(17) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943, p. 99. In the presence of bulky trityl groups, the γ-carboxyl group of N-trityl-L-glutamic acid has been coupled selectively with ethyl S-trityl-L-cysteinyglycinate using dicyclohexylcarbodiimide in a synthesis of glutathione (G. Amiard, R. Heymès and L. Velluz, *Bull. soc. chim. France*, **698** (1956)). Selective activation of the α-carboxyl group of N-benzyl-DL-aspartic acid has been effected using phosgene to yield, after subsequent treatment with NH_3, N-benzyl-DL-isoasparagine (ref. 15).

(18) J. R. Vaughan, Jr., THIS JOURNAL, **73**, 3547 (1951).

gine,[6] and this led to a high proportion of the desired *p*-tosyl-L-isoasparagine isomer.

The required *p*-tosyl-L-aspartic anhydride (IV) was obtained through treatment of *p*-tosyl-L-aspartic acid with acetic anhydride at room temperature. Although *p*-tosyl-L-isoasparagine and *p*-tosyl-L-asparagine, which was synthesized also directly from L-asparagine, both had excellent crystallizing properties, separation by crystallization of the mixture of these resulting after amidation of IV proved difficult on a reasonably large scale. The separation procedure adopted involved extraction of the mixture in aqueous suspension with a small volume of ethyl acetate. The less soluble *p*-tosyl-L-asparagine remained behind in suspension, and from the ethyl acetate extract *p*-tosyl-L-isoasparagine (V) having an estimated purity of 90% was obtained in approximately 76% yield. Each isomer was thus obtained readily in this way fairly free of the other. That a rather effective separation from the normal isomer had been achieved was confirmed by countercurrent distribution data on a sample of the *p*-tosyl-L-isoasparagine. It may be noted, however, that the degree of separation of the two isomers by extraction with ethyl acetate depended in large part upon the purity of the starting anhydride, and recrystallized anhydride was therefore usually used for the amidation reaction.

The *p*-tosyl-L-isoasparagine was further purified through a second extraction or was converted directly in reasonable yield with sodium in liquid NH_3[19] to L-isoasparagine (VI) of a high degree of purity. Electrophoretic analysis in barbital buffer was useful, as in the case with isoglutamine, and indicated that the crystallized product was free of asparagine and aspartic acid. The L-isoasparagine, after recrystallization, was reconverted to the tosyl derivative, which agreed in properties with the *p*-tosyl-L-isoasparagine obtained after the extraction procedure.

Experimental[20]

Carbobenzoxy-L-isoglutamine (II).—A solution of 8 g. of carbobenzoxy-L-glutamic acid[3] and 8 ml. of triethylamine in 48 ml. of freshly distilled tetrahydrofuran, in a round-bottom flask fitted with a thermometer and stirring bar and protected with an Ascarite tube, was cooled to −40° by means of a Dry Ice–Cellosolve-bath. Isobutyl chlorocarbonate (3.76 ml.) was added dropwise with stirring while the mixture remained in the cooling bath. The temperature rose to −20° after the addition, and the mixture was held with stirring between −20 and −35° for 10 min. A stream of dry ammonia gas was then introduced at a moderate rate for 20 min. After 10 min., the resulting thick mixture was diluted with 50 ml. of ether. The mixture was allowed to warm up to room temperature. The solvent was decanted off, and the solid was resuspended in 75 ml. of fresh ether. The somewhat hygroscopic solid was dried well *in vacuo* after decantation; it was sometimes collected rapidly on a filter and dried. The material was dissolved in a small volume of water with cooling, and the solution was adjusted to *p*H 2 with concentrated HCl. The copious white precipitate that resulted was collected on the filter. It was resuspended in 20 ml. of water and again collected; wt. 7.3 g., m.p. 148–157°. The product was triturated with 22 ml. of warm ethyl acetate; wt. 6.1 g., m.p. 159–164°. Since this material did not go to a completely clear melt, it was resuspended in 20 ml. of water, and it was washed on the filter until the filtrate was free of chloride ion; wt. 4.85 g., m.p. 163–167°. One recrystallization from ethanol–hexane yielded 3.99 g. (50%), m.p. 171–172°.

For analysis, a sample was recrystallized from water; m.p. 174.5–175°, $[\alpha]^{19}D$ −6.0° (*c* 2, methanol); reported[3] m.p. 175°, 168–172°[10]; rotation reported very low.[3]

Anal. Calcd. for $C_{13}H_{16}N_2O_5$: C, 55.7; H, 5.76; N, 10.0. Found: C, 55.6; H, 5.91; N, 9.95.

L-Isoglutamine (III).—To a solution of 1 g. of carbobenzoxy-L-isoglutamine, m.p. 172–173°, in 20 ml. of ethanol was added 8 ml. of water and 1 ml. of acetic acid, and the mixture was treated with hydrogen in the presence of 0.2 g. of 5% palladium–charcoal catalyst. After the evolution of CO_2 had ceased, the catalyst was filtered off and washed well with hot water. The filtrates were concentrated to dryness, and the residue was taken up in a small volume of water. Hot ethanol was added to incipient crystallization. Cooling and filtering yielded 456 mg. (88%), m.p. 176–177° dec. Paper electrophoresis of the material in barbital buffer, *p*H 8.5, μ 0.05, using 21 volts/cm., showed after development with ninhydrin in acidified butanol a single spot located 3.6 cm. from the origin toward the anode. Under these conditions reference samples of L-glutamine, L-isoglutamine and L-glutamic acid were located at the origin, and at positions 3.6 and 5.2 cm., respectively, toward the anode. Analysis of a small second crop obtained from the hydrogenolysis of material melting at 171–172° showed minor amounts of glutamine and glutamic acid along with the isoglutamine.

The material was recrystallized from water–ethanol as dense prisms, wt. 408 mg. (78%), m.p. 180–182° dec. A second recrystallization yielded 360 mg. melting at 183.5–184° (69%).

For analysis, the substance was again recrystallized; m.p. 186–6.5° dec., $[\alpha]^{22}D$ +21.2° (*c* 6.5, water); reported m.p. 175–186°,[4] m.p. 186°,[8b] $[\alpha]^{22}D$ +21.1° (*c* 6.5, water),[3] $[\alpha]^{21}D$ +20.5° (*c* 6.1, water).[8b]

Anal. Calcd. for $C_5H_{10}N_2O_3$: C, 41.1; H, 6.90; N, 19.2. Found: C, 40.9; H, 6.91; N, 19.2.

***p*-Toluenesulfonyl-L-aspartic Anhydride (IV).** ***p*-Tosyl-L-aspartic acid** was prepared according to the procedure for *p*-tosyl-L-glutamic acid.[8a] The product obtained through extraction of the acidified reaction mixture with ethyl acetate was crystallized from a small volume of water as the hydrate. Filtration was carried out in the cold room. A recrystallized sample melted at 86–89°, $[\alpha]^{21.5}D$ +3.4° (*c* 0.8, water); reported[21] weakly dextrorotatory in water.

Anal. Calcd. for $C_{11}H_{13}NO_6S \cdot H_2O$: C, 43.3; H, 4.95; N, 4.59; H_2O, 5.90. Found: C, 43.4; H, 4.92; N, 4.60; H_2O, 5.84.

A solution of 8 g. of dried *p*-tosyl-L-aspartic acid in 40 ml. of acetic anhydride was allowed to stand at room temperature for 18 hr. Excess acetic anhydride and other volatile material were then removed by distillation *in vacuo*, using an oil-pump and a bath held below 40°. The product crystallized after removal of the solvent. The dense crystals were triturated with dry toluene and were collected by filtration; wt. 6.5 g., m.p. 144–146°. The product was recrystallized from dry ethylene chloride, and it melted then at 147–148°, wt. 5.3 g. (71%). Further recrystallization of a sample raised the m.p. to 149°; rotation in ethyl acetate extremely low; reported[8a] m.p. 148°.

***p*-Toluenesulfonyl-L-isoasparagine (V).**—The anhydride IV, 9.65 g., m.p. 145–146°, was added in portions during 20 minutes to a solution of 80 ml. of 1 *N* NH_3 in 95% ethanol cooled in an ice-bath. A further 5-ml. volume of the ammonia solution was added at the end of this time. After 1 hr., the clear solution was allowed to warm to room temperature. It was then concentrated *in vacuo*, and the residue was dissolved in water. The resulting solution was acidified to *p*H 1, and copious crystallization soon ensued. The solid was collected by filtration. It was then resuspended in 10 ml. of water, and the suspension was extracted with four 25-ml. portions of ethyl acetate. The latter extracts were combined and concentrated to a crystalline residue. Ether was added, and the product (A) was collected by filtration; wt. 7.75 g. (76%), m.p. 154–155°, $[\alpha]^{19}D$ −14.0° (*c* 15 mg. in 1 ml. of dimethylformamide).

(19) V. du Vigneaud and O. K. Behrens, *J. Biol. Chem.*, **117**, 27 (1937).

(20) Melting points were taken in capillaries at a rate of heating of 2° per minute and are corrected.

(21) K. Freudenberg and A. Noë, *Ber.*, **58**, 2399 (1925).

A 500-mg. sample of this material was subjected to a fifty-transfer countercurrent distribution using ethyl acetate and 2 *M* phosphate buffer (K_2HPO_4–NaH_2PO_4).[4] Analysis of the curve obtained by following the ultraviolet absorption at 265 mμ indicated a homogeneity of approximately 90%. The material was recovered from the peak (K = 0.39), and this melted after recrystallization from 5% ethanol at 155–156°. The melting point depended on the rate of heating.

For analysis, a sample of A was recrystallized repeatedly from 5% ethanol as lustrous, long prisms, m.p. 155–156°, $[\alpha]^{20}D$ −13.6° (*c* 15 mg. in 1 ml. of dimethylformamide).

Anal. Calcd. for $C_{11}H_{14}N_2O_5S$: C, 46.1; H, 4.93; N, 9.79. Found: C, 46.2; H, 4.99; N, 9.50.

The material that had remained insoluble when the aqueous suspension was extracted with ethyl acetate was also collected by filtration; wt. 1.06 g. (10%), m.p. 181–184°. There was no depression in melting point upon admixture with a reference sample of *p*-tosyl-L-asparagine, m.p. 186.5–187°, prepared through direct tosylation of L-asparagine.

The *p*-tosyl-L-isoasparagine obtained as A after extraction of the crude product with ethyl acetate was of a degree of purity suitable for the following conversion to L-isoasparagine, as well as probably for other synthetic reactions. For more complete separation from the small amount of *p*-tosylasparagine remaining in A, a second extraction, using an aqueous suspension of A, appeared sufficient. Material which melted at 155° was recovered after this procedure in 81% yield.

L-Isoasparagine (VI).—A solution of 3.0 g. of V, (A), m.p. 154–155°, in 400 ml. of liquid NH_3 was held at its boiling point, and 1.1 g. of sodium was added in portions until a lasting blue color resulted. Acetic acid (2.2 ml.) was then cautiously added, and the NH_3 was allowed to evaporate. The white residue was dissolved in a small volume of warm water, the solution was adjusted to approximately *p*H 5, and boiling ethanol was added to incipient cloudiness. While still hot, the solution was rapidly centrifuged, removing the amorphous, ninhydrin-negative precipitate which had separated. On cooling, the centrifugate deposited 0.97 g. (65%) of soft needles, $[\alpha]^{20}D$ +14.7° (*c* 15 mg. in 1 ml. of 0.1 *N* HCl). The product was examined by electrophoresis on paper (Whatman No. 1), using barbital buffer, μ 0.05, *p*H 8.5 and 21 volts/cm. In this system a mixture of L-asparagine, L-isoasparagine and L-aspartic acid is well separated in 2.25 hr. After treatment of the dried paper with ninhydrin in acidified butanol, the L-asparagine is identified near the origin as a green spot, and the L-isoasparagine and L-aspartic acid are located approximately 3.5 and 5.5 cm. from the origin toward the anode as a pale reddish-blue spot and as a purple spot, respectively. A single spot characteristic of isoasparagine was obtained for the product under these conditions.

For analysis, the material was recrystallized twice as long needles from water–ethanol and was air-dried; $[\alpha]^{20}D$ +14.9°, reported[3] $[\alpha]^{18}D$ +15.5° (*c* 1.55, 0.1 *N* HCl).

Anal. Calcd. for $C_4H_8N_2O_3 \cdot H_2O$: C, 32.0; H, 6.72; N, 18.7. Found: C, 32.2; H, 6.85; N, 18.6.

A 200-mg. sample of the L-isoasparagine was reconverted to the *p*-tosyl derivative by treatment with 400 mg. of *p*-tosyl chloride in 0.8 ml. of acetone and 3 ml. of 1 *N* sodium hydroxide for 1.5 hr. On acidification of the solution 260 mg. melting at 153–154.5° was obtained. Recrystallization yielded long prisms melting at 156–156.5°.

***p*-Toluenesulfonyl-L-asparagine.**—A solution of 12.8 g. of *p*-tosyl chloride in acetone was added with stirring to a solution of 7.2 g. of L-asparagine hydrate in 55 ml. of 1 *N* sodium hydroxide. An equal quantity of 1 *N* sodium hydroxide was added in portions over the next 40 min. When after the last addition the *p*H had fallen to 7, excess *p*-tosyl chloride was removed by filtration. The clear solution was then concentrated under reduced pressure to remove acetone.

The solution was acidified to *p*H 3 and cooled. The white crystalline material which separated was collected by filtration and dried; wt. 12 g., m.p. 183–184°. The product was recrystallized once from 30% ethanol as elongated plates. Some of these were 4.5 cm. in length, wt. 10.7 g. (78%), m.p. 186.5–187°, $[\alpha]^{20}D$ −10.3° (*c* 15 mg. in 1 ml. of dimethylformamide); reported[22,23] m.p. 175°. The melting point depended on the rate of heating.

Anal. Calcd. for $C_{11}H_{14}N_2O_6S$: C, 46.1; H, 4.93; N, 9.79. Found: C, 46.4; H, 5.07; N, 9.75.

The author wishes to thank Mr. Joseph Albert for the microanalyses reported herein.

(22) S. Berlingozzi, *Gazz. chim. ital.*, **57**, 814 (1927).

(23) Very recently, a melting point of 191° (rate 4° per minute) has been reported (M. Zaoral and J. Rudinger, *Collection Czechoslov. Chem. Commun.*, **6**, 1993 (1959)).

NEW YORK, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, OKLAHOMA AGRICULTURAL EXPERIMENT STATION, OKLAHOMA STATE UNIVERSITY]

Biosynthesis of Gliotoxin. II.[1,2] Further Studies on the Incorporation of Carbon-14 and Tritium-labeled Precursors

BY J. A. WINSTEAD[3] AND R. J. SUHADOLNIK

RECEIVED AUGUST 3, 1959

Trichoderma viride incorporated DL-phenylalanine-H^3, DL-*m*-tyrosine-H^3, DL-methionine-CH_3-C^{14}, DL-serine-3-C^{14}, DL-serine-1-C^{14} and glycine-2-C^{14} into the gliotoxin, which is secreted into the medium. These results furnish additional evidence that phenylalanine is a precursor for the indole moiety. The incorporation of *m*-tyrosine indicates that hydroxylation can occur before cyclization of the aliphatic side chain of phenylalanine. The N-methyl group of gliotoxin produced from methionine-CH_3-C^{14}, serine-3-C^{14}, glycine-2-C^{14} and serine-1-C^{14} was found to contain 72, 25, 19 and 0% of the radioactivity, respectively. Radioactive gliotoxin produced from the serine-3-C^{14} was degraded to indole-2-carboxylic acid. This degradation product contained 19% of the radioactivity. All of the radioactivity incorporated into the gliotoxin from serine-1-C^{14} and 56% from serine-3-C^{14} was in carbon atoms 3, 3a and 4. A biosynthetic pathway which accounts for all 13 carbon atoms of gliotoxin is proposed.

The incorporation of phenylalanine-1-C^{14} and phenylalanine-2-C^{14} into gliotoxin by *Trichoderma viride* has been reported.[4] Alkaline degradation of gliotoxin from phenylalanine-1-C^{14} resulted in indole-2-carboxylic acid which retained 82% of the radioactivity with essentially all of the radioactivity in the carboxyl carbon. Thus, phenylalanine appeared to be a direct precursor of the indole moiety of gliotoxin. It was considered of interest to conduct further experiments to determine if the aromatic ring of phenylalanine were incorporated into

(1) This investigation was supported in part by a research grant from the United States Atomic Energy Commission, Contract No. AT(11-1)-71, Project No. 7.

(2) Presented in part at the 135th meeting of the American Chemical Society, Boston, Mass., March 1959.

(3) From the thesis submitted by Jack A. Winstead in partial fulfillment of the requirements for the Master of Science Degree at the Oklahoma State University.

(4) R. J. Suhadolnik and R. G. Chenoweth, THIS JOURNAL, **80**, 4391 (1958).