solution was extracted with four 15-ml. portions of chloroform. The solution was concentrated and chromatographed on a 4-cm. column of alumina. Bowdensine, 212 mg., was obtained in the first 150 ml. of chloroform eluent. Two successive evaporative distillations at 140° (0.50 mm.) gave pure bowdensine as a colorless glass, $[\alpha]^{24}_{589}$ +17.3°, $[\alpha]^{24}_{436}$ +48.1° (*c* 1.06, chloroform).

Anal. Calcd. for $C_{21}H_{25}NO_7$: C, 62.52; H, 6.25; N, 3.47; OCH_3, 7.69; neut. equiv., 403. Found: C, 62.48; H, 6.50; N, 3.45; OCH_3, 7.94; neut. equiv., 406.

Bowdensine Methiodide.—A solution of 45.2 mg. of bowdensine in ether was treated with an excess of methyl iodide. The methiodide, 49.4 mg., which precipitated was recrystallized from ethanol to give 41.9 mg. of bowdensine methiodide, m.p. 284–285° dec., $[\alpha]^{24}_{589}$ +9.8°, $[\alpha]^{24}_{436}$ +25.5° (*c* 1.30, 90% ethanol).

Anal. Calcd. for $C_{22}H_{28}NO_7I$: C, 48.44; H, 5.17. Found: C, 48.40; H, 5.18.

Deacetylbowdensine.—A solution of 50.8 mg. of bowdensine hydroperchlorate acetone solvate in 5 ml. of absolute ethanol containing 95 mg. of potassium hydroxide was heated under reflux for 30 minutes. The solvent was removed by evaporation and the residue was treated with a small amount of distilled water. The precipitate was removed by centrifugation and washed twice with water. Upon drying there was obtained 28.8 mg. of deacetylbowdensine, m.p. 276–277° dec., $[\alpha]^{25}_{589}$ −43.7°, $[\alpha]^{25}_{436}$ −86.6° (*c* 0.5, ethanol). Extraction of the aqueous filtrates with chloroform provided an additional 7.2 mg. of deacetylbowdensine, m.p. 260–265° dec. Recrystallization from absolute ethanol gave analytically pure deacetylbowdensine, m.p. 277–278° dec., λ^{EtOH}_{max} 287 mμ (ε 1685).

Anal. Calcd. for $C_{17}H_{21}NO_5$: C, 63.93; H, 6.63; N, 4.39; OCH_3, 9.72; neut. equiv., 319. Found: C, 64.04; H, 6.58; N, 4.27; OCH_3, 9.59; neut. equiv., 310; vicinal glycol, 0.00.

A solution of 70.7 mg. of deacetylbowdensine in absolute ethanol absorbed no hydrogen when stirred with 10% palladium-on-charcoal for 24 hours, and 68.1 mg. of starting material, m.p. 268–270° dec., was recovered.

Bowdensine from Deacetylbowdensine.—A solution of 104 mg. of deacetylbowdensine in 30 ml. of pyridine and 10 ml. of acetic anhydride was allowed to stand at room temperature for 26 hours. The solvent was removed by distillation, and the residual oil was chromatographed on Florisil. Bowdensine was eluted by 5–10% absolute ethanol in chloroform to give 113 mg. of product which showed an infrared spectrum in chloroform that was identical with that of bowdensine that had been isolated directly. A portion of the 113 mg. was evaporatively distilled at 145° (0.05 mm.) to give analytically pure bowdensine, $[\alpha]^{25}_{589}$ +17°, $[\alpha]^{25}_{436}$ +48° (*c* 1.46, chloroform).

A portion of the crude bowdensine above in ether was neutrallized with 60% perchloric acid, and the resulting precipitate was recrystallized from acetone to give bowdensine hydroperchlorate acetone solvate, m.p. 255–256° dec., which gave an infrared spectrum and optical rotation, $[\alpha]^{25}_{589}$ +5°, $[\alpha]^{25}_{436}$ +15° (*c* 0.39, 75% ethanol), identical with those of the hydroperchlorate of the natural ester.

The methiodide was prepared from a portion of the bowdensine in ether. Recrystallization from absolute ethanol gave pure bowdensine methiodide, m.p. 287–288° dec., $[\alpha]^{24}_{589}$ +11°, $[\alpha]^{24}_{436}$ +28° (*c* 1.14, 90% ethanol), the infrared spectrum of which was identical with that of the methiodide of the natural ester.

BETHESDA, MD.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, THE WEIZMANN INSTITUTE OF SCIENCE, REHOVOTH, ISRAEL]

Poly-L-cyclohexylalanine and Poly-L-cyclohexylalanyl Proteins

BY MICHAEL SELA AND RUTH ARNON

RECEIVED NOVEMBER 3, 1959

L-Cyclohexylalanine, prepared by the catalytic hydrogenation of L-phenylalanine, was allowed to react with phosgene to yield N-carboxy-L-cyclohexylalanine anhydride (III); III was polymerized in dioxane to give poly-L-cyclohexylalanine. A copolymer of L-cyclohexylalanine and L-glutamic acid was also synthesized. Poly-L-cyclohexylalanyl gelatins and poly-L-cyclohexylalanyl egg albumin, as well as a gelatin enriched with both L-cyclohexylalanine and L-glutamic acid, were obtained by making use of the amino groups of the proteins as initiators in the polymerization of N-carboxy-α-amino acid anhydrides.

Several amino acids occurring in proteins contain aromatic rings in their side chains. In studies concerning the contribution of various amino acids to specific biochemical functions of peptides and proteins, it seemed to us to be of interest to find out whether the aromatic character of the ring is necessary for the particular biological property investigated. For example, the replacement of phenylalanine by cyclohexylalanine would show the extent to which the property studied would change as a result of the saturation of the aromatic ring. Thus Jennings and Niemann[1] reported that α-chymotrypsin catalyzes the hydrolysis of acetyl-L-cyclohexylalaninamide in aqueous solutions at 25° and *p*H 7.9 at a rate equal to that of acetyl-L-phenylalaninamide. Edelson, *et al.*,[2] reported recently that cyclohexylalanine is not a phenylalanine antagonist in *Leuconostoc dextranicum* 8086.

In the course of an investigation of the chemical basis of the antigenicity of proteins[3,4] it was observed that the attachment of peptides of the aromatic amino acids tyrosine, phenylalanine and tryptophan converts gelatin into powerful antigens. In order to be able to elucidate the role of the aromatic character in the enhancement of the antigenicity of gelatin, it seemed desirable to investigate the immunological properties of derivatives of gelatin enriched with cyclohexylalanine.

The synthesis of N-carboxy-L-cyclohexylalanine anhydride (III), of poly-L-cyclohexylalanine (IV), of polycyclohexylalanyl gelatin and of other polymeric derivatives containing L-cyclohexylalanine is described in the present article. The results of the immunological studies will be reported elsewhere.

L-Cyclohexylalanine has been prepared by the catalytic hydrogenation of L-tyrosine[5-7] and of L-phenylalanine.[5] Shemin and Herbst[8] synthesized

(1) R. R. Jennings and C. Niemann, THIS JOURNAL, **75**, 4687 (1953).

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(3) M. Sela and R. Arnon, *Biochem. J.*, in press (1960).

(4) R. Arnon and M. Sela, *ibid.*, in press (1960).

(5) E. Waser and E. Brauchli, *Helv. Chim. Acta*, **7**, 740 (1924).

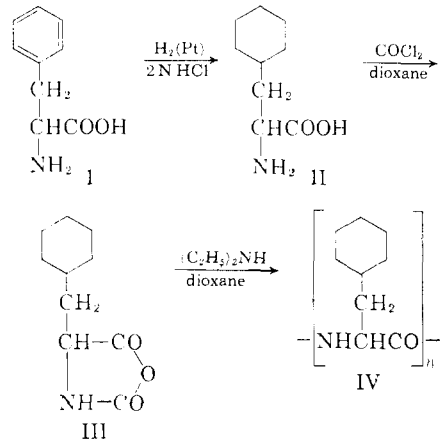
(6) P. Karrer and W. Kehl, *ibid.*, **13**, 50 (1930).

(7) J. H. Billman and J. A. Buehler, *Proc. Indiana Acad. Sci.*, **63**, 120 (1953).

(8) D. Shemin and R. M. Herbst, THIS JOURNAL, **61**, 2471 (1939).

DL-cyclohexylalanine by catalytic reduction of α-acetaminocinnamic acid, while Edelson, *et al.*,[2] obtained cyclohexylalanine upon acid hydrolysis of diethyl α-acetamido-α-cyclohexylmethylmalonate. L-Cyclohexylalanine (II) used in this study was prepared from L-phenylalanine (I) essentially according to Waser and Brauchli,[5] but the catalytic hydrogenation reaction has been carried out at a higher pressure and with much less catalyst.

Poly-L-cyclohexylalanine (IV) was synthesized according to the scheme



N-Carboxy-L-cyclohexylalanine anhydride (III) was obtained from L-cyclohexylalanine (II) and phosgene according to the general procedure of Farthing for the synthesis of N-carboxy-α-amino acid anhydrides.[9,10] The polymerization of III has been carried out in dry dioxane using diethylamine as initiator.

Poly-L-cyclohexylalanine (IV) with a number average degree of polymerization, $n = 32$, as determined by end-group analysis,[11] is soluble in dichloroacetic acid and pyridine, and insoluble in water and in most organic solvents. The structure of IV was determined by combustion analysis and by a quantitative yield of cyclohexylalanine on hydrolysis. The acid hydrolysis of IV proceeds with difficulty, in comparison with other polyamino acids, and the hydrolysis is completed with concentrated hydrochloric acid at 105° only after 48 hours.

Poly-L-cyclohexylalanine (IV) exhibits a levorotation, $[\alpha]^{20}D$ −46°, in dichloroacetic acid; IV yielded on acid hydrolysis a partially racemized cyclohexylalanine. Under similar conditions L-cyclohexylalanine (II) itself was racemized even to a greater extent. It seems, therefore, that the above synthesis of IV does not involve any change in the steric configuration.

In order to obtain a water-soluble polypeptide containing cyclohexylalanine residues in the chain, a copolymer was prepared composed of L-cyclohexylalanine and L-glutamic acid, in a residue molar ratio of 1:1. N-Carboxy-L-cyclohexylalanine anhydride was copolymerized with γ-benzyl-N-carboxy-L-glutamate anhydride,[12] and the polymerization product was debenzylated with anhydrous hydrogen bromide in glacial acetic acid.[13] The copolymer obtained had an average degree of polymerization, $n = 50$, as determined from end group analysis.[11]

Polypeptidyl proteins may be obtained by treating proteins under mild conditions (aqueous solutions, neutral *p*H, low temperatures) with N-carboxy-α-amino acid anhydrides.[14] In this way peptides of different sizes can be built onto the free α- and ε-amino groups of the original protein,[3,14-16] which thus serves as a multifunctional initiator in the polymerization of N-carboxy-α-amino acid anhydrides.[10,15,17,18] Polycyclohexylalanyl gelatin and polycyclohexylalanyl albumin have now been prepared analogously to other polypeptidyl gelatins and egg albumins.[3,4,15] The three gelatin derivatives synthesized contained, respectively, 4.4, 10.9 and 20.6% cyclohexylalanine residues. The egg albumin derivative contained 9.7% cyclohexylalanine residues. A polypeptidyl gelatin enriched with both glutamic acid and cyclohexylalanine was also prepared. It contained 19.3% glutamic acid residues (original gelatin contains 10.2% glutamic acid residues) and 18.3% cyclohexylalanine residues.

The various polypeptidyl proteins were dinitrophenylated and the hydrolyzates were analyzed for their dinitrophenylamino acid content. An authentic sample of 2,4-dinitrophenyl-L-cyclohexylalanine was also prepared. It appears that in a polycyclohexylalanyl gelatin sample containing 10.9% cyclohexylalanine residues, 23 moles of distinct polycyclohexylalanine chains were attached on the average per 100,000 g. of gelatin. The average number of cyclohexylalanine residues per peptide chain attached was, in this case, 3.5.

Experimental

All melting points are uncorrected. L-Phenylalanine and L-glutamic acid were obtained from Nutritional Biochemicals Corporation. Gelatin, U.S.P. granular, was obtained from Fisher Scientific Co. Egg albumin, twice recrystallized, was prepared according to La Rosa.[19]

L-Cyclohexylalanine Hydrochloride (II).—L-Phenylalanine (10.4 g.) was dissolved in 2 *N* hydrochloric acid (150 ml.). Platinum black (1 g., Baker and Co., Newark, N.J.) was added and the mixture hydrogenated at room temperature in a Parr bomb, starting at a pressure of 50 lb./sq. in. After 24 hours the hydrogenation was discontinued. The final pressure in the bomb amounted to 34 lb./sq. in. Most of the L-cyclohexylalanine hydrochloride formed precipitated out from the solution. It was redissolved by adding water (250 ml.) followed by warming. The catalyst was filtered off. The solution obtained, in contrast to an aqueous solution of L-phenylalanine, did not decolorize a potassium permanganate solution. It was concentrated *in vacuo* to 150 ml. and left overnight at room temperature. Crystals of III were collected and dried *in vacuo* over sulfuric acid and potassium hydroxide; yield 88%. After concentrating the mother liquor to 50 ml. a second crop of crystals was obtained, increasing the yield to 96%, m.p. 234°, $[\alpha]^{20}D$ +11.0° (*c* 3 in 1 *N* hydrochloric acid). Waser and Brauchli[5] reported m.p. 246° (cor.) and $[\alpha]^{20}D$ +11.1°.

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(17) E. Katchalski, M. Gehatia and M. Sela, *ibid.*, **77**, 6175 (1955).

(18) M. Sela, E. Katchalski and M. Gehatia, *ibid.*, **78**, 746 (1956).

(19) W. La Rosa, *Chemist Analyst*, **16**, No. 2, 3 (1927).

Anal. Calcd. for $C_9N_{18}O_2NCl$: C, 52.0; H, 8.7; N, 6.8; Cl, 17.1. Found: C, 51.8; H, 8.8; N, 7.1; Cl, 17.1.

Compound II was obtained chromatographically pure. On a chromatogram developed with *n*-butyl alcohol–acetic acid–water (50:12:50 v./v.) it shows R_f 0.86 or R_x 1.3 as compared with L-phenylalanine. In phenol–water (80:20 v./v.) the R_f value is identical with that of phenylalanine. The extinction of L-cyclohexylalanine hydrochloride, at 2580 Å., measured in aqueous solution, is zero.

2,4-Dinitrophenyl-L-cyclohexylalanine was prepared by treatment of L-cyclohexylalanine hydrochloride (0.5 g.) in aqueous solution with dinitrofluorobenzene (1 g.) for 2 hours at 40°. The reaction mixture was maintained at *p*H 9 by the addition of 25% trimethylamine in a Radiometer TTTla Autotitrator (*p*H-stat). The crude substance was isolated and purified according to Rao and Sober.[20] 2,4-Dinitrophenyl-L-cyclohexylalanine was obtained as an oil. It was further purified by chromatography in toluene–pyridine–chloroethanol–0.8 *N* aqueous ammonia, 5:1.5:3:3 v./v. The R_f value in this solvent is 0.72; the R_x value is 1.3 as compared to 2,4-dinitrophenyl-L-phenylalanine. The chromatographically purified material was eluted with dilute ammonia (0.8 *N*), dried *in vacuo* and recrystallized from acetone–benzene–petroleum ether[20]; m.p. 226°, $[\alpha]^{20}D$ −40° (*c* 2 in glacial acetic acid). The molar extinction of 2,4-dinitrophenyl-L-cyclohexylalanine in 1 *N* sodium hydroxide at 3600 Å. is 12,500.

Anal. Calcd. for $C_{15}H_{19}O_6N_3$: N, 12.5. Found: N, 12.6.

N-Carboxy-L-cyclohexylalanine anhydride (III) was prepared by passing phosgene for 2 hours through a suspension of finely ground L-cyclohexylalanine hydrochloride (5 g.) in dry dioxane (100 ml.) maintained at 50°; yield 67%, m.p. 123° (from ethyl acetate–petroleum ether) (dec. with carbon dioxide evolution). After the first recrystallization III contained less than 0.01% chlorine; $[\alpha]^{20}D$ −42.7° (*c* 5 in dry dioxane); III is readily soluble in ether, ethyl acetate, dioxane, benzene and dimethylformamide, it is insoluble in petroleum ether.

Anal. Calcd. for $C_{10}H_{15}O_3N$: C, 61.0; H, 7.6; N, 7.1; mol. wt., 197.2. Found: C, 61.1; H, 7.8; N, 7.1; neut. equiv., 198.[21]

Poly-L-cyclohexylalanine (IV).–To a solution of N-carboxy-L-cyclohexylalanine anhydride (III) (1.0 g.) in dry dioxane (25 ml.) was added diethylamine (0.02 ml.) in dioxane (0.2 ml.). Polymerization proceeded at room temperature with evolution of carbon dioxide; the solution became turbid. After 24 hours at room temperature with magnetic stirring, the polymer was precipitated with water (200 ml.), filtered and washed with water, and dried *in vacuo* over sulfuric acid; yield 0.76 g., $[\alpha]^{20}D$ −46° (*c* 5 in dichloroacetic acid). An average degree of polymerization, n = 32, was obtained by end-group analysis.[11] IV (n average 32) is soluble in dichloroacetic acid, pyridine, hot acetophenone and hot chloroform. It is sparingly soluble in dimethylformamide and glacial acetic acid. It is insoluble in water, ethanol, dioxane, ether, acetone and petroleum ether.

Anal. Calcd. for $(C_9H_{15}ON)_n$: C, 70.5; H, 9.9; N, 9.1. Found: C, 69.3; H, 9.9; N, 9.1.

Hydrolysis of Polycyclohexylalanine (IV).—Compound IV was hydrolyzed with 12 *N* hydrochloric acid in 48 hours at 105°.

A chromatographic analysis of the hydrolysate of IV, carried out as above, yielded one spot with R_f 0.86 identical with that of an authentic sample of II. The optical rotation of the dried hydrolysate was $[\alpha]^{20}D$ +7.7° (*c* 2 in 1 *N* hydrochloric acid). In order to determine whether the partial racemization occurred during the synthesis of the polymer or during the hydrolysis, the optical rotation of L-cyclohexylalanine hydrochloride (II) was measured after treatment with 12 *N* hydrochloric acid at 105° for various periods of time: 12 hours, $[\alpha]^{20}D$ +10.5°; 24 hours, $[\alpha]^{20}D$ +8.8°; 36 hours, $[\alpha]^{20}D$ +7.5°; 48 hours, $[\alpha]^{20}D$ +6.0° (*c* 2 in 1 *N* hydrochloric acid).

Copolymer of L-Cyclohexylalanine and L-Glutamic Acid.—To a solution of N-carboxy-L-cyclohexylalanine anhydride (III) (130 mg.) and γ-benzyl-N-carboxy-L-glutamate anhydride[12] (280 mg.) in dry dioxane (10 ml.) was added diethylamine (0.02 ml.). Polymerization proceeded at room temperature (magnetic stirring) with an evolution of carbon dioxide. After 24 hours the polymerization product was precipitated with water (100 ml.), filtered, washed with acetone, and dried *in vacuo* over sulfuric acid and then over phosphorus pentoxide. The dried substance was debenzylated at 2° with anhydrous hydrogen bromide in glacial acetic acid (60 hours).[13] The copolymer obtained was precipitated and washed with ether and dried *in vacuo* over potassium hydroxide and sulfuric acid.

The molar residue ratio of cyclohexylalanine and glutamic acid in the copolymer, 1.00:1.02, was determined from ninhydrin colorimetry[22] after hydrolysis in 12 *N* hydrochloric acid for 48 hours at 105° and chromatography in *n*-butyl alcohol–glacial acetic acid–water (50:12:50 v./v.). An average degree of polymerization, n = 50, was obtained by end-group analysis.[11] The copolymer is soluble in water above *p*H 5.

Polycyclohexylalanyl Gelatins.—N-Carboxy-L-cyclohexylalanine anhydride (III) (0.3 g. in 20 ml. dry dioxane) was treated with gelatin (2 g.,) in aqueous solution buffered at *p*H 7 with 0.05 *M* phosphate (50 ml.), at 5°. The reaction was carried out for 24 hours at 2°, followed by an additional 24 hours at room temperature. The reaction product was purified by dialysis against distilled water at 2° for 3 days. The contents of the dialysis bag was lyophilized and stored at 2°. The lyophilized material is insoluble in water, but well-soluble in glacial acetic acid. Dilution of solutions of the material in acetic acid with water did not cause precipitation. A chromatographic analysis of this material showed no traces of cyclohexylalanine or of low molecular weight peptides.

In order to determine the content of cyclohexylalanine, polycyclohexylalanyl gelatin was hydrolyzed with 12 *N* hydrochloric acid at 105° for 48 hours. The hydrolysate was chromatographed, after drying and redissolution in water, in *n*-butyl alcohol–glacial acetic acid–water (50:12:50 v./v.) for 24 hours. Cyclohexylalanine was determined by ninhydrin colorimetry, using the procedure of Kay, Harris and Entenman.[22] The polycyclohexylalanyl gelatin described contained 10.9% cyclohexylalanine residues. This corresponds to 80 moles of cyclohexylalanine attached per 100,000 g. of gelatin.

In order to obtain information of the extent to which the ε-amino groups of gelatin served as initiators in the polymerization of N-carboxy-L-cyclohexylalanine anhydride, polycyclohexylalanyl gelatin was dinitrophenylated at *p*H 9 at 40° for 90 min., and hydrolyzed. 2,4-Dinitrophenyl-L-cyclohexylalanine and ε,N-dinitrophenyl-L-lysine were determined spectrophotometrically.[23,24] Dinitrophenyl-L-cyclohexylalanine accounted for 74% of the amino terminal groups, while the other 26% belonged to ε, N-dinitrophenyl-L-lysine. This corresponds to 23 moles of distinct polypeptide chains per 100,000 g. of gelatin (assuming 30.6 lysine residues per 100,000 g. of gelatin[25]). Thus the average number of cyclohexylalanine residues per peptide chain attached is 3.5, in the case of the derivative described.

Two other polycyclohexylalanyl gelatins were prepared, essentially according to the same procedure, but starting, respectively, with 0.18 g. and 0.6 g. of III per 2 g. of gelatin. The derivative obtained in the first case contained 4.4% cyclohexylalanine residues, or 30 moles of cyclohexylalanine attached per 100,000 g. of gelatin. The derivative obtained in the second case contained 20.6% cyclohexylalanine residues, or 170 moles of cyclohexylalanine attached per 100,000 g. of gelatin. The last material is soluble in acetic acid, but precipitates out upon dilution with water.

Copoly-(L-glutamyl, L-cyclohexylalanyl) Gelatin.—γ-Benzyl N-carboxy-L-glutamate anhydride[12] (0.5 g. in 20 ml. of dry dioxane) and N-carboxy-L-cyclohexylalaninea nhydride (0.4 g. in 20 ml. of dry dioxane) were treated with gelatin (1.5 g.) in aqueous solution buffered at *p*H 7 with 0.05 *M* phosphate (70 ml.), at 5°. The reaction was carried out for 24 hours at 2°, followed by an additional 24 hours at room temperature. After the completion of the reaction, cold acetone (200 ml.) was added, and the precipitated gelatin derivative was washed with acetone and dried *in vacuo*, successively over sulfuric acid and phosphorus pentoxide. The

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(22) R. E. Kay, D. C. Harris and C. Entenman, *Arch. Biochem. Biophys.*, **63**, 14 (1956).

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(24) C. B. Anfinsen, M. Sela and H. Tritch, *Arch. Biochem. Biophys.*, **65**, 156 (1956).

(25) J. E. Eastoe, *Biochem. J.*, **61**, 589 (1955).

benzyl groups were removed by the action of anhydrous hydrogen bromide in glacial acetic acid at 2° for 65 hours.[13] Copoly-(L-glutamyl, L-cyclohexylalanyl) gelatin was precipitated with ether, dissolved in water at *p*H 7 (1 *N* sodium hydroxide was added for the neutralization), dialyzed against distilled water for 3 days, and lyophilized.

In order to determine the content of glutamic acid and of cyclohexylalanine the gelatin derivative described was hydrolyzed with 12 *N* hydrochloric acid at 105° for 48 hours. The hydrolysate was subjected to paper electrophoresis on Whatman No. 1 filter paper in a phthalate buffer, 0.025, *M* *p*H 5.92, at a potential gradient of 10 volts/cm., at 25°, for 2 hours. It was then chromatographed in the second dimension in *n*-butyl alcohol–glacial acetic acid–water (50:12:50 v./v.) for 48 hours. Glutamic acid and cyclohexylalanine were quantitated by ninhydrin colorimetry.[22] The gelatin derivative described contained 19.3% glutamic acid residues and 18.3% cyclohexylalanine residues. Taking into consideration that the original gelatin contained 10.1% glutamic acid residues (Eastoe[25] reports 10.2% glutamic acid residues for bone gelatin), this corresponds to an attachment of 114 moles of glutamic acid and 192 moles of cyclohexylalanine per 100,000 g. of gelatin.

Copoly-(L-glutamyl, L-cyclohexylalanyl) gelatin was dinitrophenylated and analyzed for dinitrophenylamino acids, similarly to polycyclohexylalanyl gelatins. Dinitrophenyl-L-glutamic acid accounted for 10% of the amino groups, dinitrophenyl-L-cyclohexylalanine for 43%, and ε,N-dinitrophenyl-L-lysine for 47%.

Polycyclohexylalanyl Egg Albumin.—N-Carboxy-L-cyclohexylalanine anhydride (III) (0.2 mg. in 20 ml. dry dioxane) was treated with egg albumin (1 g.) in aqueous solution buffered at *p*H 7 with 0.05 *M* phosphate (40 ml.) at 5°, and treated as described for polycyclohexylalanyl gelatin. The lyophilized final product is soluble in water. The material contained 9.7% cyclohexylalanine residues. Dinitrophenylation analysis showed that only 35% of the amino groups of the original protein served as initiators in the polymerization reaction (*i.e.*, dinitrophenyl-L-cyclohexylalanine accounted for 35% of the total dinitrophenylamino acids).

Acknowledgment.—This investigation was supported by a research grant (A-3083) from U. S. A. National Institutes of Health, Public Health Service.

[CONTRIBUTION FROM THE CHEMISTRY AND BIOLOGY DEPARTMENTS, BROOKHAVEN NATIONAL LABORATORY AND THE BOTANY DEPARTMENT, COLUMBIA UNIVERSITY]

The Biosynthesis of Nicotine from Isotopically Labeled Nicotinic Acids[1]

BY R. F. DAWSON, D. R. CHRISTMAN, A. D'ADAMO, M. L. SOLT AND A. P. WOLF

RECEIVED OCTOBER 13, 1959

The four specific ring hydrogen-labeled nicotinic acids have been prepared and fed to tobacco root cultures in sterile media, then the nicotine produced by the roots has been isolated and analyzed. Recoil tritium and carbon-14 labeled nicotinic acid have been similarly employed. The nicotine from all of these except nicotinic acid-6-*t* has shown similar and substantial incorporation into the nicotine. Oxidation of nicotinic acid, obtained from the nicotine, to the corresponding 2- and 6-pyridones has indicated that the position of hydrogen label is conserved during the conversion to nicotine. The 6-labeled acid gave less than 10% of the amount of incorporation shown by the other acids, indicating the probability of enzymatic attack on the 6-position of nicotinic acid during its conversion to nicotine by the tobacco roots. The conversion probably does not proceed *via* oxidation at the 6-position, since both 6-hydroxynicotinic acid-N^{15} and 1-methyl-6-oxynicotinamide-2-*t* failed to be incorporated. The possibility that the acid is incorporated into nicotine *via* a 1,6-dihydro intermediate is being investigated. Nicotinamide is incorporated to at least as great an extent as is the corresponding labeled acid.

Nicotinic acid was proposed as a likely precursor of nicotine in the tobacco plant by Trier[2] almost thirty years ago. At that time, there had been one reported isolation of nicotinic acid from a plant source.[3] However, the prevailing concepts of biogenesis emphasized structural relationships between natural products and those plant constituents not only of simpler nature but also of relatively widespread occurrence. Trier was led, therefore, to propose a derivation of nicotinic acid from proline (pyrrolidine-2-carboxylic acid). In his Weizmann lectures, Robinson[4] accepted the plausibility of the latter suggestion on chemical grounds, but he raised strong objections to the proposed relation between nicotinic acid and nicotine. These objections involved (a) the inertness of the 3 position of pyridine and (b) the absence of a recorded case of displacement of the nicotinic acid carboxyl group.

Following its introduction, Trier's hypothesis was subjected to repeated tests.[5] Owing to a necessary dependence upon quantitative analytical procedures and to the absence of suitable experimental systems, however, these tests yielded inconclusive results. Recently, the development of techniques for the use of isotopic tracers and for the study of nicotine production by sterile cultures of isolated tobacco roots[6] have made possible the reexamination of several hypotheses[7] including that of Trier.

The present paper offers evidence that nicotinic acid can function as a precursor of the pyridine ring of nicotine. Some chemical details of the transformation are described which eventually may afford an answer to the objections raised by Robinson.[4] Leete and others[8] have shown that proline can act as a precursor of the pyrrolidine, but not of the pyridine, ring of nicotine. We have confirmed this finding. Since, in his scheme, Trier had also

(1) Research performed under the auspices of the U. S. Atomic Energy Commission at Brookhaven and under contract No. AT (30-1)-1778 at Columbia. A grant from the Rockefeller Foundation aided the initial stages of the work.

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