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Poly-*p*-amino-DL-phenylalanine[1,2]

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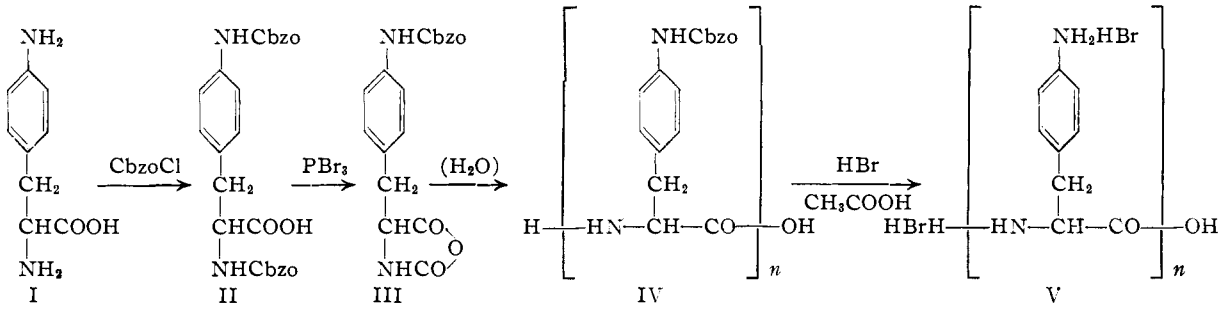
p-N-Carbobenzoxyamino-α,N-carboxy-DL-phenylalanine anhydride (III) yielded by polymerization in bulk or in solution poly-*p*-N-carbobenzoxyaminophenylalanine (IV). Poly-*p*-aminophenylalanine hydrobromide (V) was obtained by removing the carbobenzoxy groups of IV with anhydrous hydrogen bromide. Poly-*p*-nitro-DL-phenylalanine was obtained by bulk polymerization of N-carboxy-*p*-nitrophenylalanine anhydride. *p,p'*-Diamino-DL-phenylalanine anhydride was prepared from *p*-N-carbobenzoxyaminophenylalanine methyl ester. The ionization of the *p*-amino groups of *p*-aminophenylalanine and poly-*p*-aminophenylalanine has been studied by measuring the ultraviolet absorption as a function of the *p*H and ionic strength. The experimental results were found to fit the theoretical curves computed from formulas 4 and 5 assuming an intrinsic *pK* 4.55 (found for the *p*-amino group of *p*-aminophenylalanine) and radius 12.5 Å. for poly-*p*-aminophenylalanine (*n* average 15).

In continuation of experiments on water-soluble poly-α-amino acids[3-6] the synthesis of poly-*p*-aminophenylalanine is reported below. This polymer may serve as a representative of weakly basic poly-α-amino acids as well as a starting material for the preparation of polypeptidic azo dyes.

Poly-*p*-nitrophenylalanine (*n* average 100) was prepared by bulk polymerization of N-carboxy-*p*-nitrophenylalanine anhydride. Attempts to reduce this polymer to poly-*p*-aminophenylalanine, under conditions used in the reduction of *p*-nitrophenylalanine,[7,8] were unsuccessful. Poly-*p*-aminophenylalanine hydrobromide (V) was, therefore, synthesized from *p*-aminophenylalanine (I), analogously to the synthesis of polytyrosine[6] from tyrosine, as summarized in the following scheme.



Schlögl, Wessely and Korger[9] have recently reported the successful catalytic high pressure, high temperature reduction of poly-*p*-nitrophenylalanine to poly-*p*-aminophenylalanine.

As *p*-aminophenylalanine (I) yields on treatment with nitrous acid, under Van Slyke[10] conditions, one equivalent of nitrogen only, the number average degree of polymerization of V (*n* = 12 to 67) could be determined from amino-N end-group analysis.[10] Comparative amino-N end-group analysis of poly-*p*-N-carbobenzoxyaminophenylalanine (IV) and poly-*p*-aminophenylalanine hydrobromide (V) showed that no hydrolysis of peptide bonds had occurred during the removal of the carbobenzoxy groups from IV. V yields on acid hydrolysis *p*-aminophenylalanine (I) quantitatively. The absence of *p,p'*-diamino-phenylalanine anhydride in V was indicated by the negative picric acid test.[11] The dihydrobromide of this anhydride was prepared from *p*-N-carbobenzoxyaminophenylalanine methyl ester in the usual way.[12,13] The polymer V as well as *p,p'*-diamino-phenylalanine anhydride gave the corresponding azo dyes on diazotization and coupling with α-naphthol.

The change in the ionization of poly-*p*-aminophenylalanine with *p*H and ionic strength was studied spectrophotometrically in analogy to the spectrophotometric study of poly-L-tyrosine and poly-3,5-diiodotyrosine.[6] The intrinsic ionization constant of the *p*-amino group was obtained from the spectrophotometric titration of *p*-aminophenylalanine (I).

A purified sample of *p*-aminophenylalanine (I) shows at *p*H 13 between 2200 and 3000 Å. an absorption curve with two maxima at 2850 Å. (molar extinction coefficient ε 1370) and 2365 Å. (ε 9800), identical with that found by Schlögl, *et al.*[9] The ionization of the *p*-amino groups causes a considerable drop in the extinction coefficients in the whole region investigated. If the found absorption ε 45 at 2850 Å. and *p*H 1[14] is assumed for the fully ion-

(1) Presented in summary at the IInd International Congress of Biochemistry, Paris, July, 1952.

(2) Abstracted from a thesis submitted by M. Sela to the Hebrew University, Jerusalem, in partial fulfillment of the requirements for the Ph.D. degree.

(3) E. Katchalski, I. Grossfeld and M. Frankel, THIS JOURNAL, **70**, 2094 (1948).

(4) E. Katchalski and P. Spitnik, *ibid.*, **73**, 3992 (1951).

(5) A. Berger and E. Katchalski, *ibid.*, **73**, 4084 (1951).

(6) E. Katchalski and M. Sela, *ibid.*, **75**, 5284 (1953).

(7) E. Erlenmeyer and A. Lipp, *Ann.*, **219**, 213 (1883).

(8) E. D. Bergmann, THIS JOURNAL, **74**, 4947 (1952).

(9) K. Schlögl, F. Wessely and G. Korger, *Monatsh.*, **83**, 845 (1952).

(10) D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1923).

(11) E. Abderhalden and E. Komm, *Z. physiol. Chem.*, **139**, 181 (1924).

(12) E. Katchalski, I. Grossfeld and M. Frankel, THIS JOURNAL, **68**, 879 (1946).

(13) E. Katchalski and P. Spitnik, *ibid.*, **73**, 2946 (1951).

(14) The maximum absorption at *p*H 1 was found at 2570 Å., ε 360 (Schlögl, *et al.*,[9] give for 2570 Å., ε 700).

ized *p*-amino group of I, the degree of ionization α of this group at any *p*H can be calculated by means of the relation

$$\alpha = \frac{1370 - \epsilon_{2850\text{Å.}}}{1370 - 45} \quad (1)$$

The spectrophotometric titration of the *p*-amino group of I at an ionic strength of $\mu = 0.1$ is given by the open circles in Fig. 1. The ionization constant was calculated analogously to the ionization constant of aniline[15] from formula

$$p\text{H} = pK + \log \frac{1 - \alpha}{\alpha} - 0.5\sqrt{\mu}/(1 + \sqrt{\mu}) \quad (2)$$

The value obtained $pK = 4.55$ closely approaches that reported for aniline,[15] namely, 4.64 ± 0.05.

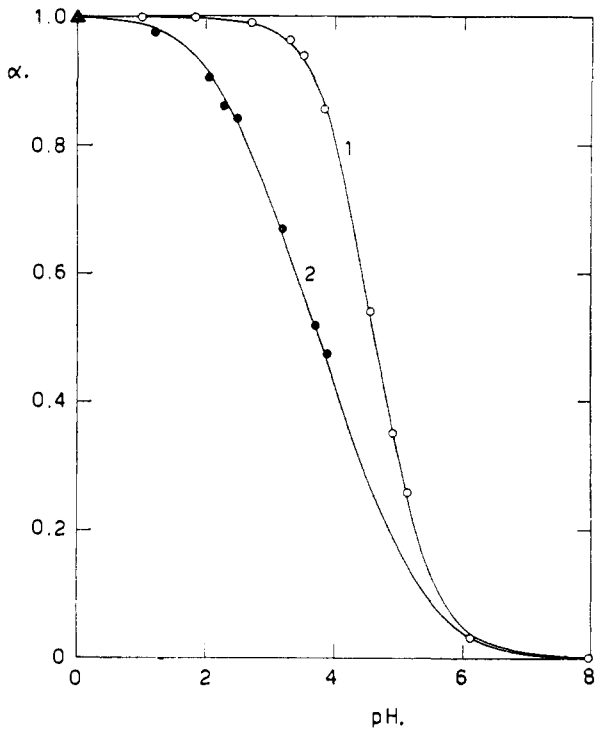


Fig. 1.—Spectrophotometric titrations of *p*-aminophenylalanine and poly-*p*-aminophenylalanine (*n* average 15) at an ionic strength of 0.1 (sodium acetate–hydrochloric acid buffers): O, experimental values for *p*-aminophenylalanine; ●, experimental values for poly-*p*-aminophenylalanine (*n* average 15); ▲, this point was obtained in *N* hydrochloric acid. Curve 1 was computed from formula 2; curve 2 was computed from formulas 4 and 5.

As poly-*p*-aminophenylalanine is insoluble in water and alkali, it was impossible to determine directly the absorption spectrum of the un-ionized polymer. It was assumed that the molar extinction coefficient per *p*-aminophenylalanine residue of the polymer at 2850 Å. is identical with the molar extinction coefficient ϵ 1370 of I at the same wave length. This assumption was supported by the observation that the molar extinction coefficient ϵ 1370, per *p*-aminophenylalanine residue of the polymer (*n* average 15) at 2850 Å., in 90% ethanolamine, was found identical with that of the monomer in the same solvent. For poly-*p*-aminophenylalanine (*n* average 15) at *p*H 1, where it may be assumed that the *p*-amino groups of the polymer are fully ionized, we found ϵ 520 per monomer residue, at 2850 Å. Different polymeric preparations gave identical absorptions at this *p*H. The reason for the considerably higher absorption of the ionized polymer as compared with the ionized monomer is obscure.[16] The degree of ionization of the *p*-amino groups of poly-*p*-aminophenylalanine at the different *p*H values investigated was calculated by means of the relation

$$\alpha = \frac{1370 - \epsilon_{2850\text{Å.}}}{1370 - 520} \quad (3)$$

The spectrophotometric titration of poly-*p*-aminophenylalanine (*n* average 15) at an ionic strength of 0.1 is given by the full circles in Fig. 1. The variation of the degree of ionization α with ionic strength at a constant *p*H is given by the circles in Fig. 2.

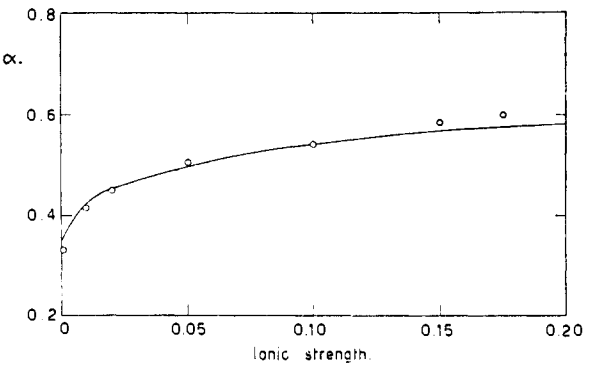


Fig. 2.—Dependence of the degree of ionization α of poly-*p*-aminophenylalanine (*n* average 15) on the ionic strength at *p*H 3.60. The desired ionic strengths were obtained by addition of sodium acetate–hydrochloric acid buffers. The full curve was computed from formulas 4 and 5.

As poly-*p*-aminophenylalanine is a polymeric base with a relatively low molecular weight, the formula 4 used by Cannan[17] and Scatchard[18] to describe the titration of various proteins may apply also in this case (*cf.* similar treatment in the case of polytyrosine[6]).

$$p\text{H} = pK_0 + \log \frac{1 - \alpha}{\alpha} - 0.868\, n\alpha w \quad (4)$$

n is the number of ionizable groups (15 in the case of poly-*p*-aminophenylalanine sample investigated) K_0, the intrinsic ionization constant, α, the degree of ionization, and *w*, the usual electrostatic interaction factor. The Debye–Hückel approximation gives

$$w = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \quad (5)$$

in which *D* is the dielectric constant of the medium, *k*, Boltzmann's constant, *T*, the absolute temperature, *e*, the electronic charge, *b*, radius of the as-

(15) L. A. Flexser, L. P. Hammett and A. Dingwall, THIS JOURNAL, **57**, 2103 (1935).

(16) The acid hydrolysate of poly-*p*-aminophenylalanine (*n* average 15) showed at *p*H 1 and 2850 Å. an extinction ϵ 130 per *p*-aminophenylalanine residue.

(17) R. K. Cannan, A. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941); R. K. Cannan, *Chem. Revs.*, **30**, 395 (1942).

(18) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

sumed sphere, $a = b + 2$ Å. = the distance of closest approach, and κ has its usual significance in the Debye theory.

Curve 2 of Fig. 1 and the full curve of Fig. 2 were computed from formulas 4 and 5 by introducing the numerical values $pK_0 = 4.55$ and $b = 12.5$ Å.

Experimental

All melting points are uncorrected. *p*-Amino-β-phenyl-DL-alanine (I) was prepared according to Bergmann.[8] Total nitrogen was determined by the Dumas method except when otherwise stated.

N,N'-Dicarbobenzoxy-*p*-aminophenylalanine (II).—Benzyl chloroformate (25 g.) and 4 *N* sodium hydroxide (40 ml.) were added simultaneously for about one hour to a solution of *p*-aminophenylalanine (11.5 g.) in 2 *N* sodium hydroxide (60 ml.). The reaction mixture was kept at 0° with vigorous stirring. After all the reagents have been added, the reaction mixture was brought to room temperature and extracted twice with 100-ml. portions of ether. The aqueous layer was acidified to congo red with 4 *N* hydrochloric acid and the N,N'-dicarbobenzoxy-*p*-aminophenylalanine, which separated out, extracted with two portions of 100 ml. of ether. The combined extracts were dried over anhydrous sodium sulfate, concentrated *in vacuo* and the crystalline solid recrystallized from benzene; yield 25.7 g. (90%), m.p. 168°.

Anal. Calcd. for $C_{25}H_{24}N_2O_6$: C, 66.9; H, 5.4; N, 6.3; neut. equiv., 448.5. Found: C, 67.2; H, 5.5; N, 6.4; neut. equiv., 450, determined by titration in ethanol with aqueous sodium hydroxide, using phenolphthalein as indicator.

II is soluble in acetone, dioxane, ethyl acetate, chloroform and boiling benzene. It is insoluble in water, in carbon tetrachloride and in petroleum ether.

***p*-N-Carbobenzoxyamino-α,N-carboxyphenylalanine anhydride** (III) was prepared from II and phosphorus tribromide in dioxane according to the general procedure of Ben-Ishai and Katchalski[19]; yield 88%, m.p. 145° (dec. with carbon dioxide evolution). Upon heating III with 3 *N* hydrochloric acid, carbon dioxide was evolved and a clear solution obtained.

Anal. Calcd. for $C_{18}H_{16}N_2O_5$: C, 63.5; H, 4.7; N, 8.2. Found: C, 63.3; H, 4.9; N, 8.0.

III is readily soluble in dioxane, ethyl acetate and acetophenone. It is insoluble in benzene and in petroleum ether.

***p*-N-Carbobenzoxyaminophenylalanine** was prepared from III in analogy to the preparation of ε,N-carbobenzoxylysine[20]; yield 96%, m.p. 230°.

Anal. Calcd. for $C_{17}H_{18}N_2O_4$: C, 65.0; H, 5.8; N, 8.9. Found: C, 65.2; H, 5.7; N, 9.1.

***p*-N-Carbobenzoxyaminophenylalanine methyl ester hydrochloride** was prepared from III in analogy to ε,N-carbobenzoxylysine methyl ester hydrochloride[9]; yield 94%, recrystallized from methanol–ether, m.p. 176°.

Anal. Calcd. for $C_{18}H_{21}N_2O_4Cl$: C, 59.3; H, 5.8; N, 7.7; CH_3O, 8.5; Cl, 9.7. Found: C, 59.1; H, 5.8; N, 7.5; CH_3O, 8.8; Cl, 10.1.

***p*-N-Carbobenzoxyaminophenylalanine Methyl Ester (VI).**—To an ice-cold solution of *p*-N-carbobenzoxyaminophenylalanine methyl ester hydrochloride (7 g.) in water (10 ml.) one equivalent of 2 *N* sodium hydroxide was added. The solution was extracted with ether, saturated with anhydrous potassium carbonate and extracted again. The combined ethereal extracts (100 ml.) were dried over sodium sulfate, the solvent removed *in vacuo* and the crystalline residue (5.8 g.) recrystallized from ether; m.p. 104°.

Anal. Calcd. for $C_{18}H_{20}N_2O_4$: C, 65.8; H, 6.1; N, 8.5; neut. equiv., 328.4. Found: C, 66.0; H, 6.3; N, 8.7; neut. equiv., 326, determined by titration in ethanol with ethanolic hydrochloric acid, using methyl orange as indicator.

VI is soluble in ethanol, ethyl acetate and ether and insoluble in water.

***p,p'*-N,N'-Dicarbobenzoxyaminophenylalanine Anhydride (VII).**—VI (2 g.) was heated for 24 hours at 120° (sealed tube) and the solid formed was washed with anhydrous ether; yield 1.3 g. (72%), recrystallized from a large volume of boiling ethanol, m.p. 247°.

Anal. Calcd. for $C_{34}H_{32}N_4O_6$: C, 68.9; H, 5.4; N, 9.4; amino-N, 0.0; carboxyl-N, 0.0; mol. wt., 592.6. Found: C, 68.3; H, 5.4; N, 9.5; amino-N, 0.0[10]; carboxyl-N, 0.04[21]; mol. wt. (Rast), 615.

VII is soluble in boiling acetic acid. It is insoluble in water and in ether. An aqueous suspension of the anhydride gives a positive picric acid test[11] and a negative ninhydrin reaction.

***p,p'*-Diaminophenylalanine Anhydride Dihydrobromide (VIII).**—VII (1.2 g.) was dissolved in a 33% solution of anhydrous hydrogen bromide in glacial acetic acid (10 g.) at room temperature.[22] The vigorous evolution of gas was accompanied by the precipitation of VIII. After 30 minutes anhydrous ether (100 ml.) was added and the precipitate separated by centrifugation, washed with anhydrous ether, dissolved in 70% ethanol (5 ml.) and reprecipitated with ether. The hygroscopic substance was dried *in vacuo* over phosphorus pentoxide and potassium hydroxide; yield 0.9 g. (91%).

Anal. Calcd. for $C_{18}H_{22}N_4O_2Br_2$: N, 11.5; α-amino-N, 0.0; Br, 32.9. Found: N, 11.3; amino-N, 0.05[10]; Br (Volhard), 32.8.

p,p'-Diaminophenylalanine anhydride dihydrobromide (VIII) is soluble in water and ethanol. The free anhydride is precipitated from an aqueous solution of VIII by adding aqueous ammonia. VIII gives a positive picric acid test[11] and a negative ninhydrin reaction. A deep red color is obtained when VIII is diazotized and coupled with α-naphthol.

The picrate of *p,p'*-diamino-phenylalanine anhydride was prepared from an aqueous solution of VIII; m.p. 195° dec.

Anal. Calcd. for $C_{30}H_{26}N_{10}O_{16}$: C, 46.0; H, 3.3; N, 17.9; Br, 0.0; α-amino-N, 0.0. Found: C, 46.7; H, 3.6; N, 17.7; Br, 0.0; amino-N, 0.0.[10]

Poly-*p*-N-carbobenzoxyaminophenylalanine (IV). (a) **By Bulk Polymerization.**—Twice recrystallized *p*-N-carbobenzoxyamino-α,N-carboxyphenylalanine anhydride (III) was heated to 150–160° in a high vacuum (10^{-4} mm.) for two hours (*cf.* preparation of polycarbobenzoxylysine[3]). The polymer obtained was dissolved in hot dichloroacetic acid and precipitated with water; yield quantitative.

Anal. Calcd. for IV (*n* average 67): C, 68.8; H, 5.5; N, 9.5; amino-N, 0.071; carboxyl-N, 0.0. Found: C, 68.4; H, 5.4; N, 9.6; amino-N, 0.071[10]; carboxyl-N, 0.0.[21]

In a preliminary experiment it was found that 2.10 g. of III yields on heating to 150° at normal pressure, 1.83 g. of IV. The amount of carbon dioxide evolved was 0.27 g. (99% of the theoretical).

In parallel polymerization experiments preparations of IV with *n* average 12, 15, 20 and 40 were obtained.

Poly-*p*-N-carbobenzoxyaminophenylalanine (IV) (*n* average 67) is soluble in hot dichloroacetic acid, in hot phenol and sparingly soluble in hot dimethylformamide. It is insoluble in glacial acetic acid, nitrobenzene, acetophenone and the usual organic solvents. On heating an aqueous suspension of IV with ninhydrin, the polymer turns deep blue, while the water remains colorless. The aqueous suspension of IV gives a negative picric acid test.[11]

(b) **By Polymerization in Solution.**—A solution of III (1.2 g.) in acetophenone (50 ml.) was heated to 120° for 48 hours. During this time part of the polymer separated out. The reaction mixture was cooled to room temperature and petroleum ether (150 ml.) was added. The precipitate was separated by centrifugation and washed several times with petroleum ether. Further purification of the polymer was obtained by dissolution in hot dichloroacetic acid and precipitation by pouring the clear solution into cold water. The supernatant was decanted and the precipitate washed several times with water and dried *in vacuo* over sulfuric acid and potassium hydroxide; yield 0.9 g. (83%).

Anal. Calcd. for IV (*n* average 20); N, 9.4; amino-N, 0.23. Found: N, 9.4; amino-N, 0.23.[10]

(19) D. Ben-Ishai and E. Katchalski, THIS JOURNAL, **74**, 3688 (1952).

(20) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

(21) D. D. Van Slyke, R. T. Dillon, D. A. MacFadyen and P. Hamilton, *ibid.*, **141**, 627 (1941).

(22) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).

Poly-*p*-aminophenylalanine Hydrobromide (V).—Poly-*p*-N-carbobenzoxyaminophenylalanine (*n* average 67) (3 g.) was dissolved in a 33% solution of anhydrous hydrogen bromide in glacial acetic acid (15 g.) at room temperature.[22] The vigorous evolution of gas was accompanied by the precipitation of V. After 30 minutes 150 ml. of anhydrous ether was added and the precipitate separated by centrifugation, washed with anhydrous ether, dissolved in 70% ethanol (10 ml.), reprecipitated with ether and dried *in vacuo* over phosphorus pentoxide and potassium hydroxide; yield 2.2 g. (89%).

Anal. Calcd. for poly-*p*-aminophenylalanine hydrobromide (V) (*n* average 67): C, 44.2; H, 4.6; N, 11.5; Br, 33.2; α-amino-N, 0.086; carboxyl-N, 0.0. Found: C, 43.8; H, 4.9; N, 11.1; Br, 33.4; amino-N, 0.087,[10] carboxyl-N, 0.0.[21]

V (*n* average 67) is soluble in water, aqueous acids and ethanol. Its aqueous solution gives a positive ninhydrin reaction, a strong biuret reaction and a negative picric acid test.[11] A deep red color is obtained when V is diazotized and coupled with α-naphthol.

V (*n* average 67) was hydrolyzed in 6 *N* hydrochloric acid in a sealed tube at 110–120° for 24 hours. The hydrolysate was evaporated *in vacuo* over sulfuric acid and potassium hydroxide and the solid residue was dissolved in water. In the hydrolyzate the amounts of amino-N,[10] carboxyl-N[21] and *p*-aminophenylalanine contents were determined, in the last case by ultraviolet absorption.

Anal. Calcd. for a hydrolyzate of 100 mg. of poly-*p*-aminophenylalanine hydrobromide (V) (*n* average 67): α-amino-N, 5.8 mg.; carboxyl-N, 5.8 mg., *p*-aminophenylalanine, 74.1 mg. Found: amino-N, 5.8 mg.[10]; carboxyl-N, 5.5 mg.,[21] *p*-aminophenylalanine, 75 mg. (calculated from the extinction of the hydrolyzate at 2850 Å. and *p*H 13 as compared with the found molar extinction coefficient ε 1370 of an authentic sample of *p*-aminophenylalanine under similar conditions).

For comparison, a parallel analysis of an authentic sample of *p*-aminophenylalanine was carried out.

Anal. Calcd. for *p*-aminophenylalanine: α-amino-N, 7.8; carboxyl-N, 7.8. Found: amino-N, 7.6[10]; carboxyl-N, 7.8.[21]

When a drop of hydrolyzate was run on a descending paper chromatogram, using 1-butanol–glacial acetic acid–water (4:1:5) as the mobile phase, one spot was obtained after spraying with ninhydrin, with R_f 0.12, identical with that of an authentic sample of *p*-aminophenylalanine run on the same strip.

Poly-*p*-aminophenylalanine Hydriodide was obtained by the treatment of IV (*n* average 15) with phosphonium iodide in dichloroacetic acid (*cf.* preparation of polylysine hydriodide[3]); yield 91%. Poly-*p*-aminophenylalanine hydriodide readily dissolves in water, aqueous acids and ethanol. Its aqueous solution gives a positive ninhydrin reaction, a strong biuret reaction and a negative picric acid test.[11]

Anal. Calcd. for poly-*p*-aminophenylalanine hydriodide (*n* average 15): C, 36.1; H, 3.4; N, 9.3; I, 45.2; α-amino-N, 0.31; carboxyl-N, 0.0. Found: C, 37.0; H, 3.8; N, 9.2; I (Volhard) 44.1; amino-N, 0.32[10]; carboxyl-N, 0.0.[21]

A hydrolyzate of poly-*p*-aminophenylalanine hydriodide (*n* average 15) prepared analogously to that of V, yielded *p*-aminophenylalanine quantitatively.

The picrate of poly-*p*-aminophenylalanine (*n* average 15) was prepared from an aqueous solution of poly-*p*-aminophenylalanine hydriodide; m.p. 180° dec.

Anal. Calcd. for poly-*p*-aminophenylalanine picrate (*n* average 15) assuming 16 picric acid molecules per polymer molecule: C, 45.4; H, 3.3; N, 17.9; I, 0.0. Found: C, 46.7; H, 3.6; N, 17.4; I (Volhard), 0.0.

The picric acid content of the picrate was determined by titration in dimethylformamide with sodium methoxide in benzene–methanol, using thymol blue as indicator.[23]

Anal. Calcd. for 100 mg. poly-*p*-aminophenylalanine picrate: picric acid, 59.9 mg. Found: picric acid, 57.1 mg.

The titration of aniline picrate under similar conditions yielded the calculated amount of picric acid.

The assumption made for poly-*p*-aminophenylalanine picrate (*n* average 15) that all the *p*-amino groups as well as the α-amino terminal group of the polymer participate in salt formation is analogous to that made for the various poly-*p*-aminophenylalanine hydrohalides investigated. A slightly better agreement with the analytical data found for poly-*p*-aminophenylalanine picrate could be obtained assuming 15 picric acid molecules per each molecule of the polymer.

Poly-*p*-aminophenylalanine hydrochloride was prepared from the picrate (*cf.* polylysine hydrochloride[3]); yield 97%.

Anal. Calcd. for poly-*p*-aminophenylalanine hydrochloride (*n* average 15): N, 14.1; Cl, 18.5; α-amino-N, 0.46. Found: N, 13.2; Cl, 18.4; amino-N, 0.43.[10]

The discrepancy between the calculated total nitrogen and the found value is probably the result of the generally known combustion difficulty of polyamides.

Poly-*p*-aminophenylalanine separated out from an aqueous solution of V (*n* average 67) upon addition of an excess of aqueous ammonia. It was purified by dissolving in hot dimethylformamide and precipitating with water.

Anal. Calcd. for poly-*p*-aminophenylalanine (*n* average 67): N, 17.2. Found: N (Kjeldahl), 16.4.

Poly-*p*-aminophenylalanine (*n* average 67) is soluble in ethanolamine, pyridine and hot dimethylformamide. It is sparingly soluble in boiling glacial acetic acid and insoluble in water, ethanol, dioxane, benzene and chloroform.

N-Carbobenzoxy-*p*-nitrophenylalanine was prepared from *p*-nitrophenylalanine[7] and benzyl chloroformate in the usual way; 92% yield, recrystallized from carbon tetrachloride, m.p. 152°.

Anal. Calcd. for $C_{17}H_{16}N_2O_6$: C, 59.3; H, 4.7; N, 8.1; neut. equiv., 344.3. Found: C, 59.4; H, 4.7; N, 7.8; neut. equiv., 348, determined by titration in ethanol with aqueous sodium hydroxide, using phenolphthalein as indicator.

N-Carbobenzoxy-*p*-nitrophenylalanine is soluble in ether, dioxane and hot benzene. It is insoluble in water and in petroleum ether.

N-Carboxy-*p*-nitrophenylalanine anhydride was prepared from N-carbobenzoxy-*p*-nitrophenylalanine and phosphorus pentachloride in anhydrous dioxane, in analogy to the preparation of ε,N-carbobenzoxy-α,N-carboxylysine anhydride[20]; yield 95%; recrystallized from dioxane–petroleum ether; m.p. 178° (dec. with carbon dioxide evolution) (Schlögl, Wessely and Korger[9] give m.p. 183 to 184°).

Anal. Calcd. for $C_{10}H_8N_2O_5$: C, 50.9; H, 3.4; N, 11.9. Found: C, 50.9; H, 3.7; N, 12.2.

Poly-*p*-nitrophenylalanine was prepared from N-carboxy-*p*-nitrophenylalanine anhydride by bulk polymerization at 180–190°, analogously to polycarbobenzoxylysine.[3] The polymer was dissolved in hot nitrobenzene and precipitated with petroleum ether; dried *in vacuo* over phosphorus pentoxide; yield quantitative.

Anal. Calcd. for poly-*p*-nitrophenylalanine (*n* average 100): C, 56.0; H, 4.2; N, 14.5; amino-N, 0.072. Found: C, 56.4; H, 4.3; N, 14.6; amino-N, 0.072.[10]

In parallel polymerization experiments preparations with *n* average 20 and 75 were obtained.

Poly-*p*-nitrophenylalanine (*n* average 100) is soluble in ethanolamine, pyridine, dimethylformamide and hot nitrobenzene. It is insoluble in water, butylamine, glacial acetic acid, *p*-cresol, chloroform, dioxane and ethanol. On heating an aqueous suspension of poly-*p*-nitrophenylalanine with ninhydrin, the polymer turns red while the water remains colorless. An aqueous solution of *p*-nitrophenylalanine turns red on heating with ninhydrin.

Poly-*p*-nitrophenylalanine (*n* average 100) was hydrolyzed in 10 *N* hydrochloric acid in a sealed glass tube at 120° for 24 hours. The hydrolyzate was evaporated *in vacuo* over sulfuric acid and potassium hydroxide and the solid residue dissolved in water. When a drop of the hydrolyzate was run on a descending paper chromatogram, using 1-butanol–glacial acetic acid–water (4:1:5) as the mobile phase, one spot was obtained after spraying with ninhydrin, with R_f 0.45, identical with that of an authentic sample of *p*-nitrophenylalanine run on the same strip.

Measurement of *p*H.—Measurements of *p*H were made on a Model G Beckman *p*H meter. A standard phosphate buffer (*p*H 7.00) was used for calibration.

Ultraviolet Light Absorption.—Measurements were made on a Beckman model DU spectrophotometer, at approxi-

(23) J. S. Fritz, *Anal. Chem.*, **24**, 306 (1952).

mately 25°. One-centimeter quartz cells were used and the extinction coefficients per *p*-aminophenylalanine residue, ϵ, calculated from the equation

$$\epsilon = (1/cd) \log_{10} (I_0/I)$$

where I_0 is the intensity of the light emerging from the solvent, I the intensity of the light emerging from the solution, c the molar concentration of the *p*-aminophenylalanine residues, and d the thickness of the absorption cell in centimeters.

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The Syntheses, Paper Chromatography and Substrate Specificity for Tyrosinase of 2,3-, 2,4-, 2,5-, 2,6- and 3,5-Dihydroxyphenylalanines[1]

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The above amino acids have been synthesized from a variety of intermediates prepared by the Erlenmeyer reaction on the appropriate dihydroxy- or dimethoxybenzaldehydes. This required the preparation of 2,6- and 3,5-dimethoxybenzaldehyde which have now been obtained in good yields in relatively large amounts by convenient procedures. The Erlenmeyer reaction on 2,6-dimethoxybenzaldehyde produced two geometric isomeric azlactones. The paper chromatographic properties of all of the dihydroxyphenylalanines are described for a number of solvent systems, in one of which the resolution of the 2,3-, 2,4-, 2,5- and 3,5-dihydroxy amino acids has been accomplished. The substrate specificity of these amino acids for the enzyme tyrosinase has been studied. 2,4-Dihydroxyphenylalanine is a reversible inhibitor of tyrosine.

As a direct result of the observation of the toxic effect of 2,5-dihydroxyphenylalanine on *Escherichia coli* (ATCC 9637) made by Volcani,[2] and because of our interest in the chromatography and enzymatic specificity shown by the amino acids, the syntheses of 2,3-, 2,6- and 3,5-dihydroxyphenylalanines was undertaken. Since these studies also required 2,4- and 2,5-dihydroxyphenylalanine, these were also synthesized, the former from three intermediates which had not been used previously in its synthesis, and the latter from 5-acetoxy-2-hydroxybenzaldehyde, a new aldehyde not previously known. 3,4-Dihydroxyphenylalanine was available from commercial sources. Samples of these dihydroxyphenylalanines have been sent to Dr. Volcani for a continuation of his studies of amino acid metabolism in bacteria.

Barltrop[3] condensed 2-hydroxy-3-methoxybenzaldehyde with benzoylglycine, acetic anhydride and sodium acetate to obtain a product which melted at 203°. He mistakenly assumed this product to be 4-(2-hydroxy-3-methoxybenzylidene)-2-phenyl-5-oxazolone on the basis of its light yellow color. The reaction actually leads to the formation of two compounds, 4-(2-acetoxy-3-methoxybenzylidene)-2-phenyl-5-oxazolone and 3-benzamido-8-methoxycoumarin. If one attempts to purify the product by recrystallization from acetic acid as Barltrop did, it is very difficult to obtain the coumarin in pure form. Fractional crystallization of the products of the reaction from benzene produces the coumarin as white crystals melting at 207–208°. Barltrop's procedure is the best one we have found for the preparation of the coumarin.

Clemo and Duxbury[4] reported their results of the above reaction on 2-hydroxy-3-methoxybenzaldehyde in which they obtained a product referred to as 4-(2-acetoxy-3-methoxybenzylidene)-2-phenyl-5-oxazolone, obtained by recrystallization from ethanol and which melted at 156–157°. If the reaction product is fractionated from benzene one does obtain the above azlactone, but in its pure form it melts at 171–172°, and the composition of the mixture of azlactone and coumarin varies depending on whether one uses 2-hydroxy-3-methoxybenzaldehyde or 2-acetoxy-3-methoxybenzaldehyde, and on the heating period. These details are presented in the Experimental section.

Clemo and Duxbury were able to convert their mixture of azlactone and coumarin to 2,3-dihydroxyphenylalanine with no difficulty since both result in the same end-product under the conditions of the reaction used. They obtained a 71% yield of amino acid which melted at 265°. Starting with pure 4-(2-acetoxy-3-methoxybenzylidene)-2-phenyl-5-oxazolone, 3-benzamido-8-methoxycoumarin and 4-(2,3-dimethoxybenzylidene)-2-phenyl-5-oxazolone, we obtained yields of 82, 90 and 87%, respectively, of material of comparable purity. When the amino acid is pure these yields are reduced to 55, 66 and 55%, respectively, of material which melted at 280° dec.

Both Hirai[5] and Deulofeu[6] have reported the synthesis of 2,4-dihydroxyphenylalanine from 5-(2,4-dimethoxybenzylidene)-hydantoin. Hirai converted the hydantoin directly to the amino acid by means of hydriodic acid and red phosphorus to obtain a product in 47% yield which melted at 223–224°. Deulofeu converted the benzylidene hydantoin to the amino acid by a longer route involving sodium amalgam reduction and finally hydriodic acid hydrolysis. He did not report his yield but stated his product was the same as Hirai's and that it melted at 223–224°. We have synthesized 2,4-dihydroxyphenylalanine from 4-(2,4-diacetoxybenzylidene)-2-phenyl-5-oxazolone, 7-acetoxy-3-benzamidocoumarin and 2-benzamido-3-(2,4-dimethoxyphenyl)-propionic acid in yields of 16, 50 and 52%, respectively, and in all cases, when the product is pure it melts at 260–262° dec.

(1) Supported in part by a Grant-in-Aid from the American Medical Association, Council on Pharmacy and Chemistry, Grant number 659.

(2) B. E. Volcani, *J. Biol. Chem.*, **192**, 543 (1951).

(3) J. A. Barltrop, *J. Chem. Soc.*, 958 (1946).

(4) G. R. Clemo and F. K. Duxbury, *ibid.*, 1795 (1950).

(5) K. Hirai, *Biochem. Z.*, **177**, 449 (1926).

(6) V. Deulofeu, *Ber.*, **69**, 2456 (1936), and a report of the same work in *Rev. orasil. chem.. Sao Paulo*, **10**, 389 (1940).