[CONTRIBUTION FROM THE WEIZMANN INSTITUTE OF SCIENCE]

The Synthesis and Spectrophotometric Study of Poly-L-tyrosine and Poly-3,5-diiodotyrosine[1]

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O-Carbobenzoxy-N-carboxy-L-tyrosine anhydride (III) yielded by polymerization in bulk or in solution poly-O-carbobenzoxy-L-tyrosine (IV). Poly-L-tyrosine (V) was obtained by removing the carbobenzoxy groups of IV with anhydrous hydrogen bromide. Iodination of V yielded poly-3,5-diiodotyrosine (VI). The ionization of the phenolic hydroxyl groups of V and VI 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 3 and 4 assuming an intrinsic pK 9.5 and radius 17.5 Å. for poly-L-tyrosine (n average 30) and intrinsic pK 7.7 and radius 26 Å. for poly-3,5-diiodotyrosine (n average 35). The results obtained suggest that all the hydroxyl groups of V and VI are free to ionize.

Introduction

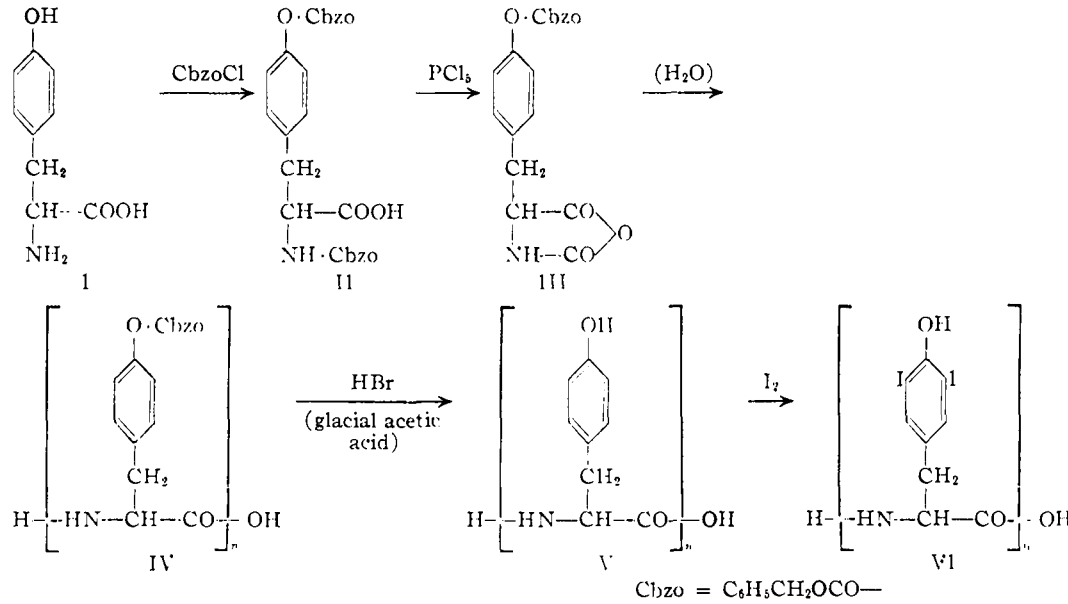
Owing to the reactivity and the ease of detection of the tyrosine residue in the protein molecule it was decided to synthesize a tyrosine polymer as a macromolecular model for the study of the behavior of tyrosine in proteins.

Low molecular peptides containing only tyrosine residues have been prepared[2] by the standard methods of peptide synthesis. However, only polymerization techniques can be used for the synthesis of tyrosine peptides possessing relatively high average molecular weight. Graziani[3] has reported the preparation of an amorphous "tyrosine anhydride" obtained by heating tyrosine in glycerol, which appears to be a tyrosine polycondensate of undefined constitution, analogous to Balbiano's[4] "glycine anhydride." Astbury, *et al.*,[5] mention the preparation of polytyrosine by polymerization, but no details of the synthesis or structure were given.

The synthesis of poly-L-tyrosine reported here was based on our experience in the synthesis of poly-α-amino acids[6] from N-carboxyamino acid anhydrides. A suitable starting monomer for this synthesis is O-carbobenzoxy-N-carboxy-L-tyrosine anhydride (III), containing a reversibly protected phenolic hydroxyl group.[7]

Results and Discussion

Synthesis.—The synthesis of poly-L-tyrosine is summarized in the scheme



Cbzo = $C_6H_5CH_2OCO$—

L-Tyrosine (I) yielded N,O-dicarbobenzoxy-L-tyrosine (II) on coupling with benzyl chloroformate. The steric configuration of II was proved by the quantitative recovery of L-tyrosine on treatment with anhydrous hydrogen bromide.[8] The O-carbobenzoxy-N-carboxy-L-tyrosine anhydride (III) was obtained from II by treatment with phosphorus pentachloride. On polymerization in bulk or in benzene solution III gave poly-O-carbobenzoxy-L-tyrosine (IV) with a number average degree of polymerization n = 20 to 80, as determined by

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

(2) A. E. Barkdoll and W. F. Ross, THIS JOURNAL, **66**, 951 (1944).

(3) F. Graziani, *Atti accad. Lincei Roma*, [5] **25**, I, 509 (1916); *Chem. Zentr.*, **87**, II, 226 (1916).

(4) L. Balbiano and D. Trasciatti, *Ber.*, **33**, 2323 (1900).

(5) W. T. Astbury, C. E. Dalgliesh, S. E. Darmon and G. B. B. M. Sutherland, *Nature*, **162**, 596 (1948).

(6) E. Katchalski, *Advances in Protein Chemistry*, **6**, 123 (1951).

(7) After this work had been completed the synthesis of a racemic polytyrosine by J. Noguchi, *Chem. High Polymers (Japan)*, **6**, 196 (1949), was brought to our attention through *C. A.*, **46**, 2605 (1952). O-carbomethoxy-N-carboxy-tyrosine anhydride was used as the starting monomer in this synthesis.

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

amino-N end-group analysis.[9] The conversion of IV to poly-L-tyrosine (V) was achieved either by use of anhydrous hydrogen bromide in glacial acetic acid[8] or by alkaline hydrolysis. Comparative amino-N end-group analysis showed that no hydrolysis of peptide bonds had occurred.

Poly-L-tyrosine (V) gave positive ninhydrin and biuret reactions. A negative picric acid test[10] indicated the absence of tyrosine anhydride[11] in V. The presence of the free phenolic groups was shown by a positive Millon reaction and by the formation of polymeric azo dyes upon coupling V with diazonium salts of various aromatic amines. Polymeric dyes of this type are now being investigated in this Laboratory.

The structure of V was determined by combustion analysis and by a quantitative yield of tyrosine on hydrolysis. The tyrosine in the hydrolysate has been determined by amino-N,[9] carboxyl-N,[12] Folin–Millon analysis[13] and by ultraviolet absorption.[14] The structure of V was further confirmed by potentiometric titration of this poly-α-amino acid in butylamine with sodium methoxide.[15] The titration showed that V contains the required number of phenolic groups.

Poly-L-tyrosine (V) yielded on acid hydrolysis a partially racemized tyrosine. Under similar conditions L-tyrosine itself was racemized even to a greater extent. It seems therefore that the above synthesis of V does not involve any change in the steric configuration.

On iodination, under conditions similar to those given for the preparation of iodinated insulin,[16] V yielded poly-3,5-diiodotyrosine (VI). The constitution of the iodinated polytyrosine was ascertained by elementary analysis and ultraviolet absorption. The molar extinction coefficient per diiodotyrosine residue at 3120 Å. and *p*H 12.0 was found identical with that of diiodotyrosine under similar conditions. The substitution of the 3- and 5-positions of the phenol residues in V is supported by the negative Millon reaction given by VI. In spite of the 3,5-substitutions VI, like 3,5-diiodotyrosine, may be coupled with the diazonium salts of various aromatic amines.[17]

Spectrophotometric Study.—The absorption spectrum of poly-L-tyrosine (V) (*n* average 30) between 2200 and 3200 Å., at several *p*H values in the alkaline region, is given in Fig. 1. The aqueous solutions used were prepared by dissolving V in aqueous sodium hydroxide of the desired strength. The absorption could not be determined below *p*H 10, V being insoluble under these conditions. The molar extinction coefficients ϵ were calculated per tyrosine residue. Figure 1 shows that at *p*H 13.6, where it may be assumed that all of the phenolic groups are ionized, two characteristic peaks, at 2420 Å. (ϵ 9800) and at 2935 Å. (ϵ 2130) are present. These two peaks correspond to those found for tyrosine and the tyrosine residue in peptides and proteins. Beaven and Holiday[14] give for tyrosine ϵ 11.050 at 2400 Å. and ϵ 2330 at 2935 Å.

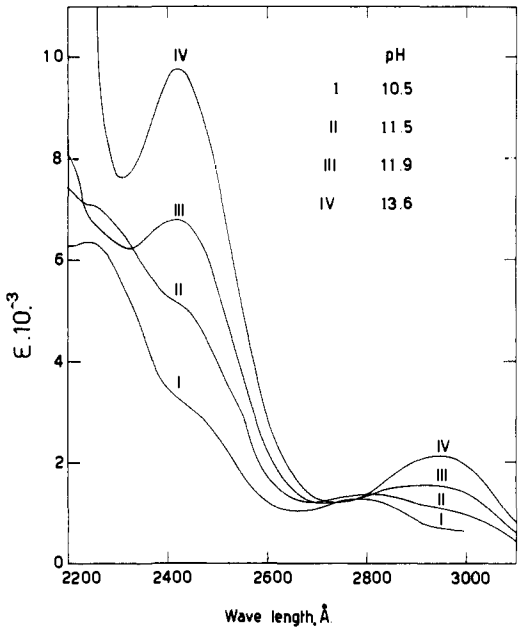


Fig. 1.—The ultraviolet absorption spectrum of poly-L-tyrosine (*n* average 30) at various *p*H values in the alkaline region.

The difference between the extinction of the tyrosine residue in V as compared with that of tyrosine itself, may be due either to the incomplete dissociation of V at *p*H 13.6 or to a slight change in the extinction of the ionized tyrosine residue when present in the polymeric chain. A similar change in the extinction was found for the tyrosine residue in insulin.[18] Assuming that polytyrosine is completely ionized at *p*H 13.6, the ϵ values given in Fig. 1 for this *p*H represent the real molar extinction for the phenolate ion of the tyrosine residue. The absorption of V at both peaks at *p*H 13.6 was found to obey Beer's law.

The different absorption spectra at the various *p*H values given in Fig. 1 are due to the different ratios of the ionized to the un-ionized tyrosine residues. For tyrosine at *p*H 2.0 we found ϵ 300 at 2420 Å. and ϵ 30 at 2935 Å. If this absorption is assumed for the un-ionized tyrosine residue of polytyrosine, the degree of ionization α of the phenolic hydroxyls of V at any *p*H can be calculated by means of either of the two relations

$$\alpha = \frac{\epsilon_{2420\text{Å}} - 300}{9500} \quad (1); \qquad \alpha = \frac{\epsilon_{2935\text{Å}} - 30}{2100} \quad (2)$$

Identical values of α were obtained from both formulas. It is thus possible to carry out a spectropho-

(9) D. D. Van Slyke, *J. Biol. Chem.*, **83**, 425 (1929).
(10) E. Abderhalden and E. Komm, *Z. physiol. Chem.*, **139**, 181 (1924).
(11) E. Fischer and W. Schrauth, *Ann.*, **354**, 35 (1907).
(12) D. D. Van Slyke, R. T. Dillon, D. A. MacFadyen and P. Hamilton, *J. Biol. Chem.*, **141**, 627 (1941).
(13) O. Folin and V. Ciocalteu, *ibid.*, **73**, 636 (1927).
(14) G. H. Beaven and E. R. Holiday, *Advances in Protein Chemistry*, **7**, 319 (1952).
(15) J. S. Fritz and N. M. Lisicki, *Anal. Chem.*, **23**, 589 (1951).
(16) C. R. Harington and A. Neuberger, *Biochem. J.*, **30**, 809 (1936).
(17) W. Koment, *Biochem. Z.*, **321**, 93 (1950); *Z. physiol. Chem.*, **286**, 229, 235 (1951).
(18) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

tometric titration of V analogously to that of tyrosine and of proteins.[14,18-22]

None of the solutions showed any change in spectrum on standing, nor did urea have any effect. The entire spectrophotometric titration was reversible, so that V does not appear to be subject to denaturation. It may thus be assumed that all the tyrosine hydroxyl groups in the polytyrosine molecule are completely free to ionize. In this respect V resembles insulin,[18] lysozyme,[21] bovine[22] and human[23] serum albumin, but not egg albumin.[18]

The spectrophotometric titration of poly-L-tyrosine (n average 30) at an ionic strength of 0.2 is given by the open circles in Fig. 2. The variation

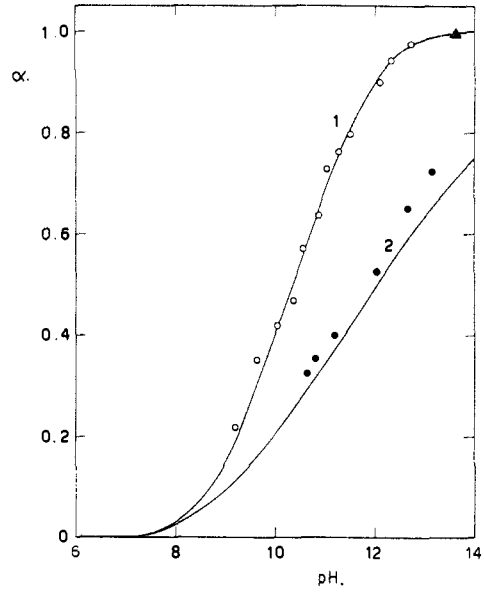


Fig. 2.—Spectrophotometric titrations of poly-L-tyrosine (n average 30): O, experimental values at an ionic strength of 0.2 (NaCl); ●, experimental values obtained by direct titrations with sodium hydroxide in the absence of salt, extrapolated to zero ionic strength; ▲, this point was obtained at pH 13.6. Curves 1 and 2 were computed from formula 3 for the corresponding ionic strengths.

of the degree of ionization α with ionic strength at a constant pH is given by the circles in Fig. 3.

As polytyrosine is a polymeric acid, its titration may presumably be described by the theoretical treatment used for polyelectrolytes.[24] However, since no data are available as to the change in the shape of the molecule during the titration, and since V (n average 30) is a relatively small molecule, it was assumed as a first approximation that the formula (3) used by Cannan[25] and Scatchard[26]

(19) W. Stenström and N. Goldsmith, *J. Phys. Chem.*, **30**, 1683 (1926).

(20) J. W. Sizer and A. C. Peacock, *J. Biol. Chem.*, **171**, 767 (1947).

(21) C. Fromageot and G. Schnek, *Biochem. Biophys. Acta*, **6**, 113 (1950).

(22) C. Tanford and G. L. Roberts, Jr., THIS JOURNAL, **74**, 2509 (1952).

(23) C. Tanford, *ibid.*, **72**, 441 (1950).

(24) A. Katchalsky, O. Künzle and W. Kuhn, *J. Polymer Sci.*, **5**, 283 (1950).

(25) 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).

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

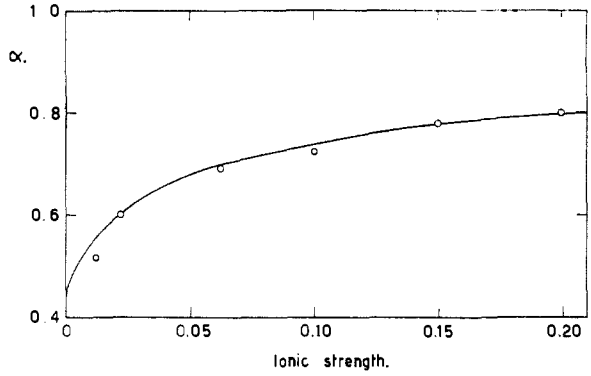


Fig. 3.—Dependence of the degree of ionization, α, of poly-L-tyrosine (n average 30) on the ionic strength at pH 11.5. The full curve was computed from formula 3.

to describe the titration of various proteins may also apply in this case.

$$p\mathrm{H} = pK_0 - \log \frac{1-\alpha}{\alpha} + 0.868n\alpha w \qquad (3)$$

n is the number of ionizable groups (30 in the case of polytyrosine sample investigated), K_0 is the intrinsic ionization constant, α is the degree of ionization, and w is the usual electrostatic interaction factor.

The Debye–Hückel approximation gives for a sphere of constant radius, b, with a charge spread uniformly on its surface

$$w = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) \qquad (4)$$

in which D is the dielectric constant of the medium, k is the Boltzmann constant, T is the absolute temperature, e is the electronic charge, a is the distance of closest approach, and κ has its usual significance in the Debye theory.

Curve 1 of Fig. 2 which fits closely the experimental data at 0.2 ionic strength was computed from formula 3 by introducing the numerical values pK_0 9.5 and w 0.0658. The intrinsic ionization constant used approaches closely the pK_0 9.6 estimated by Tanford and Roberts[22] as the intrinsic constant of the phenolic groups of tyrosine. From the above value for w the approximate radius for the assumed sphere, b = 17.5 Å., and the distance of closest approach, $a = b + 2$ Å. = 19.5 Å., were calculated from formula 4 for a polytyrosine molecule with an average molecular weight of 5000. This value seems reasonable when compared with the radius of 30 Å. for serum albumin of a molecular weight of 69000.[27] The constant, w, can be evaluated for various ionic strengths from the calculated radius with the aid of formula 4. By using these values the theoretical curve in Fig. 3 was computed. The calculated curve fits the experimental data closely and thus independent support is provided for the general assumptions made.

Curve 2 of Fig. 2 computed by using the above assumptions gives the spectrophotometric titration of V at zero ionic strength. The full circles represent measurements of direct titration with sodium hydroxide in the absence of salt, extrapolated to zero ionic strength according to formula 3. The

(27) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 186 (1947).

theoretical curve does not coincide exactly with the extrapolated experimental points and a better agreement could be obtained by increasing b (*cf.* Katchalsky, *et al.*[24]).

The ultraviolet absorption spectrum of polydiiodotyrosine (n average 35) was determined at several pH values (Fig. 4) analogously to that of polytyrosine. The molar extinction coefficients ϵ were calculated per diiodotyrosine residue. At pH 11.9 where it may be assumed that all of the hydroxyl groups are ionized, a characteristic peak is present at 3120 Å. (ϵ 5460). This wave length as well as the corresponding molar extinction coefficient were practically identical with those found by us for the characteristic peak of diiodotyrosine at the same pH (ϵ 5400 at 3120 Å.).[28]

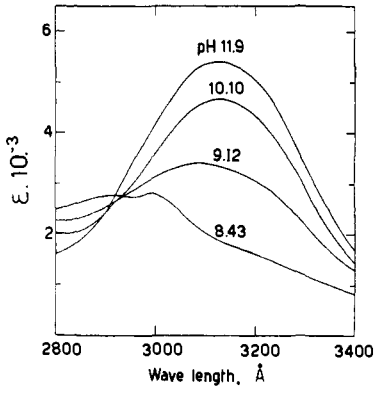


Fig. 4.—The ultraviolet absorption spectrum of poly-3,5-diiodotyrosine (n average 35) at various pH values in the alkaline region.

As diiodotyrosine at pH 1.0 gives at 3120 Å., ϵ 160, the degree of ionization α of the hydroxyl groups of polydiiodotyrosine at any pH can be calculated from the relation

$$\alpha = \frac{\epsilon_{3120\text{Å}} - 160}{5300} \qquad (5)$$

In experiments analogous to those carried out with polytyrosine it was shown that all the hydroxyl groups in polydiiodotyrosine are free to ionize. The spectrophotometric titration of polydiiodotyrosine (n average 35) at the ionic strengths 0.01 and 0.2 is given in Fig. 5. Figure 6 gives the degree of ionization as a function of the ionic strength at a constant pH. The full curves given in both figures were calculated from formula 3 by assuming pK_0 7.7 and w 0.034. From the above value for w the approximate radius for the assumed sphere b = 26 Å., and the distance of closest approach, $a = b +$ 2 Å. = 28 Å., were calculated from formula 4 for a polydiiodotyrosine molecule with an average molecular weight of 14500. The titration curves of VI show that iodinated polytyrosine is a much stronger polyacid than polytyrosine itself. However, the intrinsic ionization constant used is considerably lower than that (pK 6.48) found for diiodotyrosine by Dalton, *et al.*[29] By assuming a lower pK_0 in formula 3, a considerable discrepancy between the experimental data and the theoretical curve would occur. From the data given in the literature[23,30,31] no final conclusion about the intrinsic ionization constant of the iodinated tyrosine residues in proteins could be drawn. The b calculated is considerably larger than that chosen for polytyrosine. This is in accord with the increase in molecular weight after iodination.

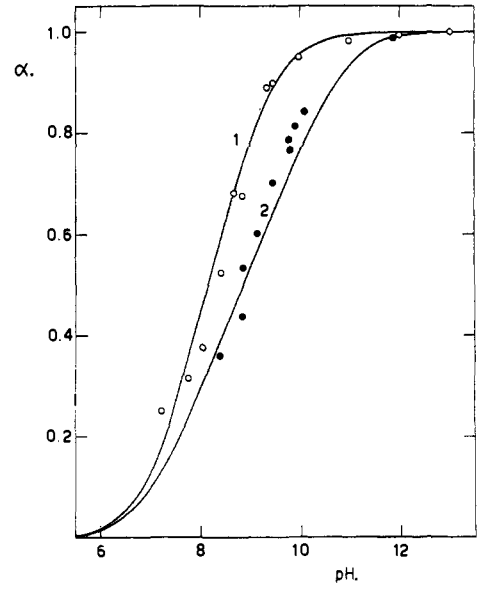


Fig. 5.—Spectrophotometric titrations of poly-3,5-diiodotyrosine (n average 35): O, experimental values at an ionic strength of 0.2; ●, experimental values at an ionic strength of 0.01. The desired ionic strengths were obtained by addition of glycine or phosphate buffers. Curves 1 and 2 were computed from formula 3 for the corresponding ionic strengths.

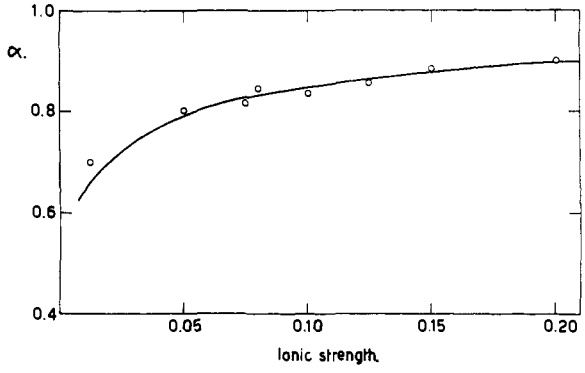


Fig. 6.—Dependence of the degree of ionization, α, of poly-3,5-diiodotyrosine (n average 35) on the ionic strength at pH 9.5. The full curve was computed from formula 3.

Curve 2 of Fig. 5 does not coincide exactly with the experimental points and a better agreement could be obtained by increasing b (analogously to curve 2 of Fig. 2).

Experimental

All melting points are uncorrected. Tyrosine obtained from Nutritional Biochemicals Corporation was used throughout.

(28) L. A. Ginsel, *Biochem. J.*, **33**, 428 (1939), found ϵ_{max} 5600 for 3100 Å. Crammer and Neuberger[18] report two maxima, one at 3160 Å. (ϵ 5260) and the other at 3060° Å. (ϵ 5170).

(29) J. B. Dalton, P. L. Kirk and C. L. A. Schmidt, *J. Biol. Chem.*, **88**, 589 (1930).

(30) A. Neuberger, *Biochem. J.*, **28**, 1982 (1934).

(31) E. J. Cohn, W. T. Salter and R. M. Ferry, *J. Biol. Chem.*, **123**, XXIV (1938).

N,O-Dicarbobenzoxy-L-tyrosine (II).—Benzyl chloroformate (25 g.) and 4 *N* sodium hydroxide (40 ml.) were added simultaneously for about one hour to a solution of L-tyrosine (11.5 g.) in 2 *N* sodium hydroxide (60 ml.). The reaction mixture was kept at 0° with vigorous stirring. The *p*H was maintained between 9 and 11, by adjusting the rate of flow of the benzyl chloroformate and the sodium hydroxide. After all the reagents had 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,O-dicarbobenzoxytyrosine, 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 residue twice recrystallized from carbon tetrachloride. II was thus obtained in long colorless needles; yield 25.3 g. (89%), m.p. 117°, $[\alpha]^{20}D$ −5° (*c* 10 in glacial acetic acid).

Anal. Calcd. for $C_{25}H_{23}NO_7$: C, 66.8; H, 5.2; N, 3.1; neut. equiv., 449.4. Found: C, 66.7; H, 5.4; N, 3.0; neut. equiv., 451, determined by titration in ethanol with aqueous sodium hydroxide, using phenolphthalein as indicator.

II is soluble in ether, ethyl acetate, dioxane, methanol, ethanol, boiling carbon tetrachloride and boiling benzene. It is insoluble in water and in petroleum ether. The Millon reaction of II is negative. Abderhalden and Bahn[32] reported the synthesis of an amorphous N,O-dicarbobenzoxytyrosine, but no detailed characterization of this material was given.

Regeneration of L-Tyrosine from N,O-Dicarbobenzoxy-L-tyrosine (II).—II (2.47 g.) was mixed with 12 g. of a 33% solution of anhydrous hydrogen bromide in glacial acetic acid[8] at room temperature. Gas evolution started immediately, and tyrosine hydrobromide separated out within 15 minutes. After two hours the precipitation was brought to completion by the addition of 30 ml. of anhydrous ether. The supernatant solution was decanted and the precipitate washed several times with anhydrous ether; yield of tyrosine hydrobromide, 1.41 g. (98%).

Anal. Calcd. for $C_9H_{12}NO_3Br$: amino-N, 5.3; Br, 30.5. Found: amino-N, 5.3[9]; Br, 30.6 (Volhard).

From the optical rotation of a solution of tyrosine hydrobromide (*c* 14 in 4% hydrochloric acid), $[\alpha]^{20}D$ −11.5° for the tyrosine derived from II was calculated. An authentic sample of L-tyrosine under similar conditions gave $[\alpha]^{20}D$ −11.8° (*c* 10 in 4% hydrochloric acid) (*cf.* also Stein, *et al.*[33]).

N,O-Dicarbobenzoxy-DL-tyrosine was prepared from DL-tyrosine analogously to the L-derivative; recrystallized from benzene, m.p. 127°. Admixture with N,O-dicarbobenzoxy-L-tyrosine (II) caused a depression in m.p. to 106°.

Anal. Calcd. for $C_{25}H_{23}NO_7$: C, 66.8; H, 5.2; N, 3.1; neut. equiv., 449.4. Found: C, 67.0; H, 5.3; N, 3.3; neut. equiv., 449, determined by titration in ethanol with aqueous sodium hydroxide, using phenolphthalein as indicator.

N-Carbobenzoxy-DL-tyrosine was prepared by coupling equimolar amounts of DL-tyrosine and benzyl chloroformate; yield 80%, m.p. 124° (m.p. of N-carbobenzoxy-L-tyrosine, according to Bergmann and Zervas,[34] 101°).

Anal. Calcd. for $C_{17}H_{17}NO_5$: C, 64.8; H, 5.4; N, 4.4; neut. equiv., 315.3. Found: C, 65.0; H, 5.4; N, 4.5; neut. equiv., 318, determined by titration in ethanol with aqueous sodium hydroxide, using phenolphthalein as indicator.

N-carbobenzoxy-DL-tyrosine gives a positive Millon reaction.

O-Carbobenzoxy-N-carboxy-L-tyrosine Anhydride (III).—II (6 g.) was dissolved in boiling anhydrous benzene (60 ml.). Ten ml. of solvent was distilled azeotropically to remove traces of moisture. The solution was quickly cooled to 0° and 3.1 g. of phosphorus pentachloride added. The reaction mixture was shaken for five minutes and the small residue of unchanged phosphorus pentachloride filtered off. The clear solution was heated to 60° for five minutes to enhance intramolecular cyclization of the relatively stable chloride.[32] After three hours at room temperature, 150 ml. of petroleum ether was added, the supernatant fluid was decanted and the semi-solid precipitate redissolved in benzene and reprecipitated with petroleum ether. The crystalline anhydride thus obtained was thoroughly washed with petroleum ether and dried *in vacuo* over sulfuric acid and potassium hydroxide; yield 4 g. (88%); recrystallized from benzene–petroleum ether, m.p. 101° (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 $C_{18}H_{15}NO_6$: C, 63.3; H, 4.4; N, 4.1; neut. equiv., 341.3. Found: C, 62.9; H, 4.7; N, 4.0; neut. equiv., 339, determined by titration in dioxane with sodium methoxide in benzene–methanol (1:1 by volume), using thymol blue as indicator.[35]

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

Poly-O-carbobenzoxy-L-tyrosine (IV). (a) By Bulk Polymerization.—Twice recrystallized, O-carbobenzoxy-N-carboxy-L-tyrosine anhydride (III) was heated to 110° in high vacuum (10^{-4} mm.) for four hours (*cf.* preparation of polycarbobenzoxylysine[36]). The polymer obtained was dissolved in hot glacial acetic acid and precipitated with water, slightly acidified with hydrochloric acid.

Anal. Calcd. for IV (*n* average 25): C, 68.5; H, 5.1; N, 4.7; amino-N, 0.19; carboxyl-N, 0.0. Found: C, 68.0; H, 5.1; N, 4.7; amino-N, 0.19[9]; carboxyl-N, 0.04.[12]

In a preliminary experiment it was found that 3.50 g. of III yield, on heating to 110° at normal pressure, 3.05 g. of IV. The amount of carbon dioxide evolved was 0.45 g. (97.4%).

In parallel polymerization experiments preparations of IV with *n* average 20, 28, 30, 35, 50, 75 and 80 were obtained.

Poly-O-carbobenzoxy-L-tyrosine (IV) (*n* average 25) is soluble in hot glacial acetic acid, dichloroacetic acid, dimethylformamide, acetophenone, chloroform, phenol, nitrobenzene and concd. sulfuric acid. 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 Millon reaction and a negative picric acid[10] test.

(b) By Polymerization in Solution.—A solution of III (1.6 g.) in anhydrous benzene (50 ml.) was refluxed for 24 hours. During this time a part of the polymer separated out. The reaction mixture was cooled to room temperature and petroleum ether (150 ml.) added. The precipitate was separated by centrifugation and washed several times with petroleum ether. Further purification of the polymer was attained 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, dried *in vacuo* over sulfuric acid and potassium hydroxide; yield 1.1 g. (79%).

Anal. Calcd. for IV (*n* average 57): C, 68.6; H, 5.1; N, 4.7; amino-N, 0.083. Found: C, 68.0; H, 5.2; N, 4.7; amino-N, 0.083.[9]

Poly-O-carbobenzoxy-L-tyrosine (*n* average 57) is only slightly soluble in boiling glacial acetic acid, while IV with a lower average degree of polymerization (*n* average 25), prepared by bulk polymerization, readily dissolves in hot glacial acetic acid.

Poly-L-tyrosine (V). (a) By Removal of the Carbobenzoxy Group from IV with Hydrogen Bromide.[8]—Poly-O-carbobenzoxy-L-tyrosine (IV) (*n* average 75) (2.4 g.) was mixed with a 33% solution of anhydrous hydrogen bromide in glacial acetic acid (15 g.) at room temperature. The polymer went into solution with the vigorous evolution of gas. After 30 minutes 150 ml. of anhydrous ether was added and the precipitate separated by centrifugation, washed with anhydrous ether, dissolved in 10 ml. of absolute ethanol and reprecipitated by pouring the ethanolic solution into ice-cold water. The flocculent colorless precipitate was centrifuged, washed with water, and dried *in vacuo* at 80° over phosphorus pentoxide and potassium hydroxide; yield 1.23 g. (93%), $[\alpha]^{20}D$ +8.7° (*c* 7 in 1 *N* sodium hydroxide).

(32) E. Abderhalden and A. Bahn, *Z. physiol. Chem.*, **219**, 78 (1933).

(33) W. H. Stein, S. Moore and M. Bergmann, THIS JOURNAL, **64**, 724 (1942).

(34) M. Bergmann and L. Zervas, *Ber.*, **65**, 1199 (1932).

(35) A. Berger, M. Sela and E. Katchalski, *Anal. Chem.*, in press.

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

Anal. Calcd. for poly-L-tyrosine (V) (*n* average 75): C, 66.1; H, 5.6; N, 8.6; amino-N, 0.115; carboxyl-N, 0.0. Found: C, 64.8; H, 5.9; N, 8.6; amino-N, 0.11[9]; carboxyl-N, 0.03.[12]

Poly-L-tyrosine (V) is soluble in aqueous alkali, alkali carbonates and in ammonia. It is readily soluble in methanol, ethanol, butylamine, ethanolamine, pyridine, aniline, phenol and *p*-cresol. It is also soluble in benzyl alcohol, glacial acetic acid, dichloroacetic acid, dimethylformamide, nitrobenzene and concentrated sulfuric acid. V is slightly soluble in acetone and chloroform and insoluble in water, aqueous alkali bicarbonate, benzene, ether and petroleum ether. It is practically insoluble in a saturated aqueous solution of lithium bromide. On heating an aqueous suspension of V with ninhydrin, the polymer turns deep blue while the water remains colorless. On heating an aqueous suspension of V with Millon reagent, the insoluble polymer turns red while the water remains colorless. V gives a strong violet biuret reaction and a negative picric acid test.[10] A deep violet color is obtained on coupling V with *p*-nitrobenzenediazonium chloride.

The phenolic hydroxyl groups of poly-L-tyrosine were titrated potentiometrically in a solution of V in freshly distilled butylamine, with sodium methoxide in benzene–methanol (1:1 by volume), using an antimony electrode against a glass electrode, according to Fritz and Lisicki.[15] From the amount of sodium methoxide added up to the inflection point of the titration curve, the equivalent weight per phenolic hydroxyl was calculated.

Anal. Calcd. for tyrosine residue $C_9H_9HO_2$: neut. equiv., 163.2. Found: neut. equiv., 166.

(b) By Removal of the Carbobenzoxy Groups from IV with Sodium Hydroxide.—Poly-O-carbobenzoxy-L-tyrosine (IV) (*n* average 25) (0.35 g.) was shaken with 1 *N* sodium hydroxide (12 ml.) for 4 hours at room temperature. Within this period a clear solution was obtained. It was acidified to congo red with 4 *N* hydrochloric acid and the precipitate formed centrifuged. The polytyrosine (V) thus obtained was washed several times with water and dried *in vacuo* at 80° over phosphorus pentoxide and potassium hydroxide; yield 0.15 g. (73%).

Anal. Calcd. for polytyrosine (V) (*n* average 25): C, 65.9; H, 5.6; N, 8.6; amino-N, 0.34. Found: C, 64.5; H, 5.8; N, 8.6; amino-N, 0.38.[9]

Total Hydrolysis of Poly-L-tyrosine (V) (*n* average 75).—V (*n* average 75) (0.1 g.) was suspended in 10 *N* hydrochloric acid (1 ml.) and the suspension kept in a sealed glass tube at 110–120° for 24 hours. The resulting dark clear solution was evaporated to dryness *in vacuo* over sulfuric acid and potassium hydroxide. The solid residue was dissolved in 5 ml. of water and the solution diluted to a volume of 100 ml. From this solution aliquots were withdrawn for the determination of amino-N,[9] carboxyl-N[12] and tyrosine content by the Folin–Millon method[13] and by ultraviolet absorption. In the last case the tyrosine content was calculated from the extinction of the hydrolysate at 2935 A. and *p*H 13 as compared with the found molar extinction coefficient ϵ 2350 of an authentic sample of L-tyrosine under similar conditions (*cf.* also Beaven and Holiday[14]).

Anal. Calcd. for a hydrolysate of 100 mg. of poly-L-tyrosine (*n* average 75): amino-N, 8.5 mg.; carboxyl-N, 8.5 mg.; tyrosine, 111 mg. Found: amino-N, 8.9 mg.[9]; carboxyl-N, 8.2 mg.[12]; tyrosine, 107 mg. (Folin[13]); tyrosine, 112 mg. (ultraviolet absorption).

When a drop of hydrolysate was run on an ascending paper chromatogram, using *n*-butyl alcohol–acetic acid–water (4:1:5) as the mobile phase, one spot was obtained by spraying with ninhydrin, with R_F 0.62, identical with that of an authentic sample of L-tyrosine run on the same strip.

All attempts to determine the optical rotation of the tyrosine hydrolysate using a sodium lamp were unsuccessful, due to the yellow color of the solution and the relatively small rotation of L-tyrosine. It was thus decided to determine the optical rotation of a tyrosine sample derived from the hydrolysate by the following method: 1 g. of polytyrosine (V) (*n* average 75) obtained from IV by method (a) was hydrolyzed under conditions similar to those given above. The solid tyrosine hydrochloride derived from hydrolysate was redissolved in 5 ml. of water and the acid solution neutralized with pyridine. Ethanol was added and the mixture left in the refrigerator overnight. The colorless tyrosine which precipitated was filtered, washed with 5 ml. of ethanol and dried *in vacuo* over phosphorus pentoxide; yield 1.05 g. (95%), $[\alpha]^{20}D$ −9.5° (*c* 8 in 4% hydrochloric acid). One-gram samples of authentic L-tyrosine were dissolved in 10 ml. of 10 *N* hydrochloric acid each and the final solution kept in sealed glass tubes at 110–120° for different time intervals. After treatment analogous to that described for the poly-L-tyrosine (V) hydrolysate the different tyrosine samples recovered showed the rotation specified in Table I. No attempt was made to carry out the hydrolysis of V under milder conditions, where a lower degree of racemization might be expected, since even in boiling concentrated hydrochloric acid the polymer is hydrolyzed slowly.

TABLE I

CHANGE IN THE OPTICAL ROTATION OF L-TYROSINE UPON TREATMENT WITH 10 *N* HYDROCHLORIC ACID AT 110–120°

Time of heating, hr.	$[\alpha]^{20}D$ (*c* 8 to 9 in 4% HCl)	Time of heating, hr.	$[\alpha]^{20}D$ (*c* 8 to 9 in 4% HCl)
0	−11.8°	16	−8.0°
4	−11.8	24	−6.0
10	− 9.5	30	−5.4

Poly-3,5-diiodotyrosine (VI).—Poly-L-tyrosine (*n* average 35) (230 mg.) was dissolved in a solution of 2 ml. of ammonium hydroxide (sp. gr. 0.90) in 15 ml. of methanol. The solution was kept at 0° and 2.7 ml. of an iodine–potassium iodide solution, prepared by dissolving 3.15 g. of iodine in 10 ml. of 40% potassium iodide, added in three portions during half an hour; 2.3 moles of iodine was thus added per mole of tyrosine residue. After an additional hour at 0°, glacial acetic acid (2 ml.) was added. The flocculent yellow precipitate of the iodinated polypeptide was centrifuged and washed several times with 5% aqueous acetic acid. VI was dissolved in dioxane, precipitated with ice-cold water containing a few drops of dilute hydrochloric acid, centrifuged, washed with water and finally dried *in vacuo* over sulfuric acid and potassium hydroxide; yield 520 mg. (88%).

Anal. Calcd. for polydiiodotyrosine (VI) (*n* average 35): C, 26.0; H, 1.7; N, 3.4; I, 61.1; amino-N, 0.096. Found: C, 26.7; H, 2.0; N, 3.6; I, 59.0; amino-N, 0.085.[9]

Polydiiodotyrosine (VI) is soluble in aqueous alkali, ethanol, dioxane and ethanolamine. It is sparingly soluble in acetone and insoluble in water, benzene and butylamine. A sample of VI dispersed in water gave a negative starch reaction for iodine. The yellow color of VI did not disappear on shaking with a dilute thiosulfate solution. An aqueous suspension of VI gives a negative Millon reaction. A deep red color is obtained on coupling VI with *p*-nitrobenzenediazonium chloride.[17]

A comparison between the measured molar extinction coefficient of the diiodotyrosine residue in polydiiodotyrosine (ϵ 5460 at 3120 Å. and *p*H 12) with the measured molar extinction coefficient of an authentic sample of diiodotyrosine (ϵ 5400) under similar conditions, confirmed the structure of iodinated polytyrosine.

Measurement of *p*H.—Measurements of *p*H were made on model G Beckman *p*H meter. A standard phosphate buffer (*p*H 7.00) was used for calibration. A Beckman Type E high *p*H glass electrode, standardized against a borate buffer (*p*H 10.00), was used for readings above *p*H 10.

Ultraviolet Light Absorption.—Measurements were made on a Beckman model DU spectrophotometer,[37] at approximately 25°. One centimeter quartz cells were used and the extinction coefficients per tyrosine—or per diiodotyrosine—residue, ϵ, calculated from the equation

$$\epsilon = \frac{1}{cd} \log_{10} \frac{I_0}{I} \qquad (6)$$

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 tyrosine or diiodotyrosine residues, and d the thickness of the absorption cell in centimeters.

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