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

Multichain Polyamino Acids

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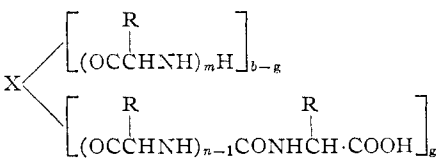
The use of polylysine or polyornithine as multifunctional initiators in the polymerization of N-carboxy-α-amino acid anhydrides yielded multichain polyamino acids composed of peptidic side chains attached to a multifunctional core. The preparation and properties of the following multichain polyamino acids are described: multi-poly-DL-alanyl-poly-L-lysine, multi-polysarcosyl-poly-L-lysine, multi-poly-L-lysyl-poly-L-lysine, multi-poly-L-lysyl-poly-DL-ornithine, multi-poly-L-tyrosyl-poly-L-lysine and multi-poly-L-glutamyl-poly-L-lysine.

All the synthetic poly-α-amino acids described in the literature[1,2] are linear. Most of them were prepared by the polymerization of N-carboxy-α-amino acid anhydrides, using amines or water as initiators of polymerization. In the present article the preparation of a new type of non-linear amino acid polymers will be described. These are the multichain polyamino acids obtained by the polymerization of N-carboxy-α-amino acid anhydrides using polyfunctional initiators.[3,4] As each functional group of the initiator may start a polymeric chain, every multichain molecule will obviously contain a number of peptidic side chains equal to the number of the functional groups of the initiator. In the absence of a termination reaction[2] each multichain molecule will contain growing chains only, characterized by terminal α-amino groups. In the presence of a termination reaction the multichain polymer will contain molecules composed of growing as well as terminated chains, the latter being characterized by terminal carboxyl groups. The general formula of a multichain polyamino acid molecule may thus be represented schematically as



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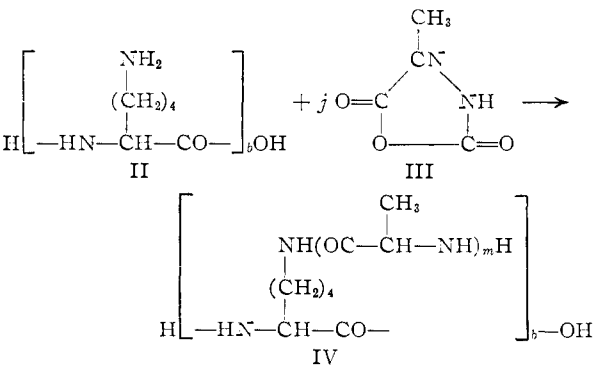
In this formula X denotes the multifunctional core, derived from the b-functional initiator, XH_b, g is the number of terminated side chains and $(b - g)$ the number of growing side chains. The numbers m and n determine the number of residues in the still growing side chains and the terminated side chains, respectively. Obviously the values of m and n may vary from side chain to side chain.

Linear poly-L-lysine[5] was used in most polymerization experiments as the multifunctional initiator. The polymerization could not, however, be performed as usual in an anhydrous medium, as it was observed that the free polylysine base is insoluble in most organic solvents. Since N-carboxy-α-amino acid anhydrides are known to react with amines considerably faster than with water[6,7] the polymerization could be carried out in aqueous media. The medium chosen for the polymerization was a 1:1 mixture of dioxane and dilute phosphate buffer *p*H 7. In all the polymerizations described a part of the N-carboxy-α-amino acid anhydride used as monomer reacted with water to give the corresponding free α-amino acids as well as short linear polypeptides. These could be separated from the required multichain polymer by prolonged dialysis.

Multi-poly-DL-alanyl-poly-L-lysine was prepared from N-carboxy-DL-alanine anhydride[8] and poly-L-lysine according to the following scheme, in which it has been assumed for simplicity that only the ε-amino groups of the polylysine served as initiating functional groups and that no termination reaction[2] took place. Under the polymerization conditions used in the preparation of the multichain DL-alanine polymer, but in the absence of polylysine, N-



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carboxy-DL-alanine anhydride gives low alanine polypeptides which passed through the dialyzing membrane to an extent of 97%. It thus seems justified to assume that a dialyzed multi-poly-DL-alanyl-poly-L-lysine preparation is practically free of linear polymer.

A considerable percentage of the polylysine (average degree of polymerization DP 36) used as initiator in the preparation of the multichain poly-DL-alanine passes through the cellophane membrane. The absence of lysine in the hydrolysate of the material removed from the multichain polyalanine on dialysis, as well as the fact that both lysine and alanine were found in the hydrolysate of the non-dialyzable material, proves therefore that

(1) E. Katchalski, *Adv. Protein Chem.*, **6**, 123 (1951).

(2) M. Sela and A. Berger, THIS JOURNAL, **77**, 1893 (1955).

(3) The term "multichain polymer" was introduced by J. R. Schaefgen and P. J. Flory, (*ibid.*, **70**, 2709 (1948)) to denote branched polymers whose molecules are composed of linear polymeric chains attached to a polyfunctional core.

(4) Preliminary notes on multichain polyamino acids have been published: M. Sela, *Bull. Research Council of Israel*, **4**, 109 (1954); M. Sela and E. Katchalski, *Experientia*, **11**, 62 (1955).

(5) E. Katchalski, I. Grossfeld and M. Frankel, THIS JOURNAL, **70**, 2094 (1948); E. Katchalski, "Methods in Enzymology," edited by S. P. Colowick and N. O. Kaplan, Vol. III, Academic Press, Inc., New York, N. Y.

(6) F. Wessely, K. Riedl and H. Tuppy, *Monatsh.*, **81**, 861 (1950).

(7) R. R. Becker and M. A. Stahmann, *J. Biol. Chem.*, **204**, 737 (1953).

(8) J. L. Bailey, *J. Chem. Soc.*, 3461 (1950).

all the polylysine used as initiator was transformed into a non-diffusible, high molecular weight multichain polymer, as a result of its interaction with N-carboxyalanine anhydride. In order to determine whether all the ε-amino groups of the polylysine reacted with N-carboxyalanine anhydride, the multichain polymer was dinitrophenylated,[9] the product hydrolyzed, and the hydrolysate analyzed chromatographically. DNP-alanine,[10] but no ε-DNP-lysine,[9] could be detected on the chromatogram. This indicates that practically all the ε-amino groups of polylysine reacted with N-carboxyalanine anhydride to form ε-amide bonds. This conclusion was supported by the observation that the hydrolysate of a desaminated multi-polyalanyl-polylysine did not contain any of the amino acids which appeared in the hydrolysate of a desaminated polylysine. The finding that all the polylysine reacted during the synthesis of the multichain polyalanine was corroborated by the fact that the incubation of the multichain polymer with trypsin did not lead to the formation of lysyllysine and lysine, the characteristic tryptic digestion products of poly-L-lysine.[1,11]

The average number of alanine residues, p, per side chain of the multichain polymer was determined by two independent methods: (a) The multi-poly-DL-alanyl-poly-L-lysine was hydrolyzed and the ratio between alanine and lysine was determined chromatographically.[12] (b) The amino and carboxyl end groups of the multichain polymer were determined, the former by Van Slyke analysis or by titration with perchloric acid,[2] while the latter by titration with sodium methoxide,[2] and the average molecular weight of the side chains was calculated similarly to that of a linear polyamino acid.[2] A good correlation was obtained between both methods (p = 25 according to (a) and p = 22 according to (b)). Since the average degree of polymerization of the polylysine used as initiator was b = 36, the average molecular weight of the multi-poly-DL-alanyl-poly-L-lysine (b 36, p 25) is 36 × [128 + (25 × 71)] = 68,508, where 128 stands for the molecular weight of a lysine residue and 71 for the molecular weight of an alanine residue. This calculation assumes that no side chain is attached to the α-amino group of the polylysine, and it omits the ureidocarbonyl groups of the terminated side chains.

Multi-poly-DL-alanyl-poly-L-lysine (b 36, p 25) may be salted out from its aqueous solution by sodium chloride or ammonium sulfate in contradistinction to the linear poly-DL-alanine (DP 18) which stays in solution. The solubility of the branched poly-DL-alanine in aqueous sodium chloride is given in Fig. 1. Paper electrophoresis experiments have shown that the multichain polymer does not move at pH 9.1, but travels at pH 3.6 as a round spot toward the cathode. A mixture of a linear polyalanine and a linear polylysine separates at pH 3.6 into a circular spot of polyalanine, remaining at the origin, and an elongated strip of polylysine directed toward the cathode.

The viscosities of a linear poly-DL-alanine (DP 18) in water and of multi-poly-DL-alanyl-poly-L-lysine (b 34, p 22) in a glycine buffer of pH 9.1 and ionic strength 0.1, at 20°, as a function of concentration are given in Fig. 2. The viscosity of the branched polymer shows a peculiar concentration dependence, similar to the one found by Batzer[13] for branched polyesters.

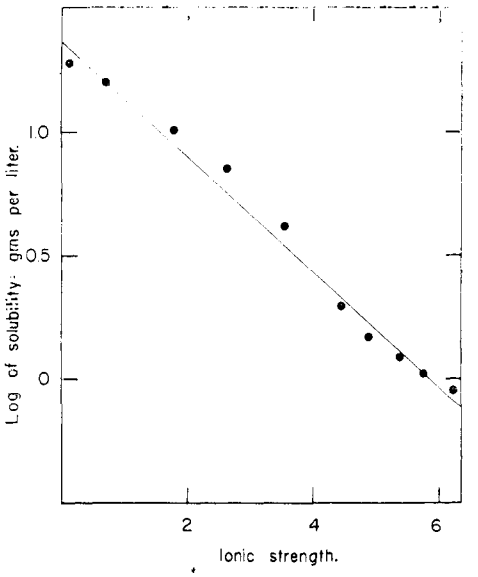

Fig. 1.—The solubility of multi-poly-DL-alanyl-poly-L-lysine (b 36, p 25) in aqueous sodium chloride.

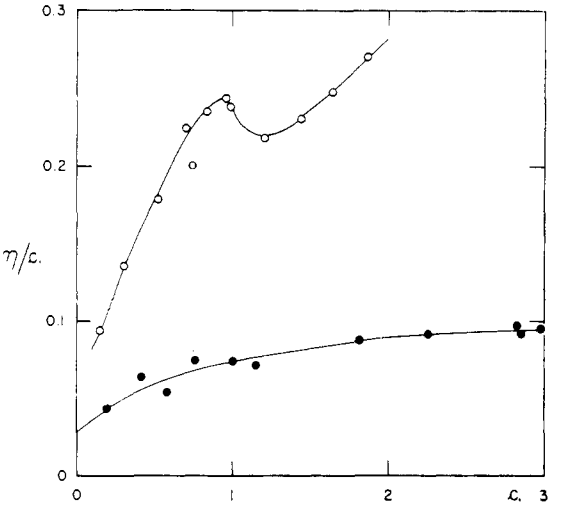

Fig. 2.—Viscosity as a function of concentration (c in grams per 100 ml.) at 20° of: ○, multi-poly-DL-alanyl-poly-L-lysine (b 34, p 22) in glycine buffer, pH 9.1, and ionic strength 0.1; ●, linear poly-DL-alanine (DP 18) in water.

The diffusion coefficient of the linear poly-DL-alanine (DP 18) in water at a concentration range of 0.5 to 1.5% was found to be practically independent of concentration and equal to $D_{20} = 28 \times 10^{-7}$ cm.²/sec. The diffusion coefficient of the multi-poly-DL-alanyl-poly-L-lysine (b 34, p 22) in a glycine buffer of pH 9.1 and ionic strength 0.1 as a function of concentration is given in Fig. 3.

The sedimentation coefficient of the multi-poly-

(9) F. Sanger, *Biochem. J.*, **39**, 507 (1945).
(10) E. Abderhalden and P. Blumberg, *Z. physiol. Chem.*, **65**, 318 (1910).
(11) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).
(12) R. A. Boissonas, *Helv. Chim. Acta*, **33**, 1975 (1950).
(13) H. Batzer, *Die makromol. Chem.*, **12**, 145 (1954).

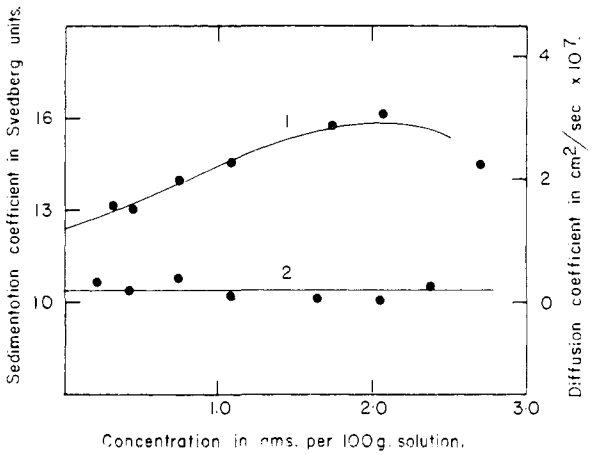

Fig. 3.—The concentration dependence of the diffusion (1) and sedimentation (2) coefficients of multi-poly-DL-alanyl-poly-L-lysine (*b* 34, *p* 22) in a glycine buffer of *p*H 9.1 and ionic strength 0.1, at 20°.

DL-alanyl-poly-L-lysine (*b* 34, *p* 22) as a function of concentration, is also given in Fig. 3. For comparison an estimate of the sedimentation coefficient of the linear poly-DL-alanine (DP 18) in water, S_{20} = 0.3 Svedberg units, was obtained from measurements in a synthetic boundary cell.[14] The relatively low diffusion and high sedimentation coefficients of the multi-polyalanyl-polylysine, as compared with the linear poly-DL-alanine, show that the branched polymer has an average molecular weight considerably higher than that of the linear polymer.

Multi-polysarcosyl-poly-L-lysine (*b* 20, *p* 7) was prepared analogously to the synthesis of multichain polyalanine, but using N-carboxysarcosine anhydride[15] as monomer. The poor yield obtained is probably the result of the rapid hydrolysis of N-carboxysarcosine anhydride in water. As it has been shown that the multichain polysarcosine does not contain free amino groups, it appears that all the ε-amino groups of the polylysine also reacted in this case to yield ε-amide bonds.

The multichain polyelectrolytes: multi-poly-L-lysyl-poly-L-lysine (*b* 30, *p* 15), multi-poly-L-lysyl-poly-DL-ornithine (*b* 36, *p* 3), multi-poly-L-tyrosyl-poly-L-lysine (*b* 200, *p* 8) and multi-poly-L-glutamyl-poly-L-lysine (*b* 200, *p* 6), were prepared by polymerization of ε,N-carbobenzoxy-α,N-carboxy-L-lysine anhydride,[16] O-carbobenzoxy-N-carboxy-L-tyrosine anhydride[17] and γ-benzyl-N-carboxy-L-glutamate anhydride,[18] respectively, in a 1:1 water–dioxane solution, using linear poly-L-lysine or poly-DL-ornithine[19] as polymerization initiators, subsequent removal of the carbobenzoxy or benzyl groups by treatment with anhydrous hydrogen bromide,[20] and dialysis. In the case of multi-poly-γ-benzyl-glutamylpolylysine the yield could be increased by carrying out the polymerization in a dioxane solution containing only 3% water.

Multi-poly-L-lysyl-poly-L-lysine (*b* 30, *p* 15) yielded on incubation with trypsin practically quantitatively lysyllysine and lysine. Since only lysine derivatives containing a free ε-amino group may be digested by trypsin,[21] the polylysine side chains were probably hydrolyzed first, while the polylysine core was attacked only after the liberation of its ε-amino groups. The formation of lysyllysine and lysine is in accord with the observation that the same products are obtained on tryptic digestion of linear poly-L-lysine.[1,11]

Multi-poly-L-lysyl-poly-DL-ornithine (*b* 36, *p* 3) yielded on incubation with trypsin lysyllysine and lysine, as well as a polypeptidic fraction containing ornithine and lysine in a molar ratio of 2 to 1. Since poly-DL-ornithine is resistant toward trypsin, it is obvious that the polyornithine core remained intact. Furthermore, as the side chains of the multi-poly-L-lysyl-poly-DL-ornithine studied were very short (*n* 3) and as it is known that L-lysine amide, L-lysyl-L-lysine and tri-L-lysine[11,22] are hydrolyzed by trypsin considerably slower than higher lysine peptides, it is not surprising that some lysine was found in the undigested peptidic fraction in combination with poly-DL-ornithine.

A statistical analysis of the molecular weight distribution of multichain polyamino acids was carried out.[23] It was shown that a sharp molecular weight distribution is to be expected in the absence of a termination reaction, and in polymers containing terminated side chains only. The relatively high molecular weight of the multichain polyamino acids synthesized, the sharp molecular weight distribution, as well as our observation that some of the branched polyamino acids are non-toxic, indicates that their possible use as blood volume extenders is worth investigating.

Experimental

Multi-poly-DL-alanyl-poly-L-lysine.—An ice-cooled solution of N-carboxy-DL-alanine anhydride[2,3] (18 g.) in anhydrous dioxane (250 ml.) was mixed with an ice-cooled solution of poly-L-lysine hydrobromide.[5] DP 36 (450 mg.) in *M*/75 phosphate buffer *p*H 7.0 (200 ml.), and the reaction mixture was left in the refrigerator overnight. The gel formed was introduced into a cellophane bag and dialyzed against water for five days at room temperature. The biuret and ninhydrin reactions of the dialysate were negative at the end of this period. The clear solution left in the cellophane bag was concentrated *in vacuo*, and the residue was triturated with acetone and dried over sulfuric acid; yield 3.8 g. The combined dialysates were similarly concentrated *in vacuo* and dried.

The non-dialyzable material was hydrolyzed in 10 *N* hydrochloric acid at 110° for 24 hours, and the hydrolysate was analyzed chromatographically on paper, using 1-butanol–pyridine–glacial acetic acid–water (15:10:3:12) as the mobile phase. Two spots, identified as alanine and lysine, were revealed after spraying with ninhydrin. A quantitative chromatographic analysis[12] showed the molar ratio of alanine to lysine to be 25 to 1. This corresponds to an average molecular weight of (25 × 71) + (1 × 128) = 1903 for each chain of the non-dialyzable multichain polymer. (The numbers 71 and 128 stand for the molecular weights of alanine and lysine residues, respectively). An

(14) H. K. Schachman and W. F. Harrington, *J. Polymer Sci.*, **12**, 379 (1954); F. Tietze and H. Neurath, THIS JOURNAL, **75**, 1758 (1953).
(15) F. Sigmund and F. Wessely, *Z. physiol. Chem.*, **157**, 91 (1926).
(16) M. Bergmann, L. Zervas and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).
(17) E. Katchalski and M. Sela, THIS JOURNAL, **75**, 5284 (1953).
(18) W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3239 (1950).
(19) E. Katchalski and P. Spitnik, THIS JOURNAL, **73**, 3992 (1951).
(20) D. Ben-Ishai and A. Berger, *J. Org. Chem.*, **17**, 1564 (1952).
(21) K. Hofmann and M. Bergmann, *J. Biol. Chem.*, **130**, 81 (1939).
(22) S. G. Waley and J. Watson, *Biochem. J.*, **57**, 529 (1954).
(23) E. Katchalski, M. Gehatia and M. Sela, THIS JOURNAL, **77**, 6175 (1955).

average molecular weight of 1680, for each chain of the multichain polymer, was calculated from end-group analysis.[2] The amino groups were determined by titration in glacial acetic acid with perchloric acid in glacial acetic acid, using crystal violet as indicator[2] (equiv. wt., 2100), and by Van Slyke analysis (amino N, 0.65). The carboxyl groups were determined by titration of a suspension in dimethylformamide with sodium methoxide in benzene–methanol, using thymol blue as indicator[2] (equiv. wt., 8500).

Both the chromatographic and end-group analyses of various multi-poly-DL-alanyl-poly-L-lysine preparations obtained by making use of poly-L-lysine samples with different average chain lengths as initiators, showed that the multichain polymers obtained had the following composition: *b* 36, *p* 40; *b* 20, *p* 25; *b* 34, *p* 20; *b* 20, *p* 15; *b* 16, *p* 21; and *b* 200, *p* 41.

In order to determine whether the multi-polyalanyl-polylysine contains free ε-amino groups of lysine the following experiment was carried out: A solution of the multichain polymer (450 mg.) in *M* sodium bicarbonate (50 ml.) was mixed with 2,4-dinitrofluorobenzene (1.0 ml.) in ethanol (10 ml.), and the reaction mixture was shaken mechanically for two hours. The suspension formed was extracted with ether, the ether layer discarded, and the aqueous suspension left brought to dryness *in vacuo* and hydrolyzed with 10 *N* hydrochloric acid at 110° for 24 hours. The hydrolysate was extracted with five portions of benzene (3 ml. each), and both the combined benzene extracts and the aqueous solution were analyzed chromatographically on paper, using toluene–chloroethanol–pyridine–0.8 *N* ammonia (10:6:3:6)[24] as the mobile phase. The benzene extract was found to contain DNP-alanine[10] with an R_f identical with that of an authentic sample. The amount of DNP-alanine determined spectrophotometrically after chromatographic separation,[24] corresponded to 45% of the free amino groups of the multichain polymer. No ε-DNP-lysine[9] could be detected chromatographically in the aqueous solution.

The absence of free ε-amino lysine groups in the multichain polymer was also demonstrated by the following deamination experiment: A solution of multi-polyalanyl-polylysine (35 mg.) in water (5 ml.) was mixed with a saturated aqueous solution of sodium nitrite (10 ml.) and glacial acetic acid (3 ml.). The reaction mixture was shaken mechanically for two hours and left overnight at room temperature. It was then dialyzed against water for three days, and the contents of the dialysis bag concentrated *in vacuo* and hydrolyzed in 10 *N* hydrochloric acid at 110° for 24 hours. The hydrolysate was brought to dryness and analyzed chromatographically using 1-butanol–pyridine–glacial acetic acid–water (15:10:3:12) as the mobile phase. After spraying with ninhydrin, two spots, identified as alanine and lysine, were obtained. The molar ratio of alanine to lysine in the hydrolysate was found to be 23 to 1.[12] When the product of desamination of a sample of linear polylysine was hydrolyzed and chromatographed under similar conditions, three spots were obtained after spraying with ninhydrin. The major spot had an R_x, 3.80, as referred to lysine, and corresponded most likely to ε-hydroxy-α-aminocaproic acid; the other two spots had R_x values of 1.34 and 1.70.

The material that passed the cellophane membrane during the dialysis of the reaction mixture containing the multichain polymer, was hydrolyzed and chromatographed similarly to the non-dialyzable material. Only one spot, identified as alanine, was revealed after spraying with ninhydrin. This proves that the dialysate contains linear polyalanine only. A linear poly-DL-alanine, obtained under conditions identical with those given for the preparation of the multichain polymer but in the absence of polylysine, yielded a material, 97% of which passed through the cellophane membrane. The polylysine (*b* 36) used as multifunctional initiator, passed through the cellophane membrane to a considerable extent.

The rapid digestion of poly-L-lysine by trypsin[1,11] was not affected by a large excess of poly-DL-alanine. A chromatographic analysis of an incubation mixture of multi-poly-DL-alanyl-poly-L-lysine with trypsin at *p*H 7.6 and 25°, showed that the branched polymer is resistant toward trypsin.

The electrophoretic mobility of multi-poly-DL-alanyl-poly-L-lysine (*b* 20, *p* 25) was compared with that of linear poly-DL-alanine (DP 25), linear poly-L-lysine (DP 20), as well as with that of a mixture of linear polyalanine (DP 25) and linear polylysine (DP 20) in a ratio of 25 alanine residues to one lysine residue. The measurements were carried out on a Whatman No. 1 filter paper in an acetate buffer of *p*H 3.6 and ionic strength 0.2, at a potential gradient of 10 volt/cm., at 25°. After two hours the polymers were revealed with ninhydrin. The linear polyalanine did not move from the origin. The linear polylysine spread into an elongated spot of 8.6-cm. length, toward the cathode. The mixture of the two linear polymers gave a round spot of polyalanine at the origin, and a thin elongated spot corresponding to polylysine. The multichain poly-DL-alanine migrated 2.8 cm. from the origin toward the cathode as one circular spot. The same multichain poly-DL-alanine migrated toward the anode in 0.1 sodium carbonate, and did not move from the origin in a borate buffer of *p*H 9.05 and ionic strength 0.1.

Multi-poly-DL-alanyl-poly-L-lysine (*b* 36, *p* 25) gives a positive ninhydrin reaction and a strongly positive biuret reaction. It is soluble in water, partially soluble in hot glacial acetic acid, and insoluble in methanol, ethanol, chloroform, dioxane, pyridine, dimethylformamide and nitrobenzene. The branched polymer is precipitated from concentrated aqueous solution by acetone, ethanol–ether, by saturated sodium chloride or ammonium sulfate. It is not precipitated from its aqueous solution by trichloroacetic acid or by ethanol.

Multi-polysarcosyl-poly-L-lysine.—An ice-cooled solution of N-carboxysarcosine anhydride[15] (6.3 g.) in dioxane (60 ml.) was mixed with an ice-cooled solution of poly-L-lysine hydrobromide,[5] DP 20 (275 mg.), in *M*/150 phosphate buffer *p*H 7 (100 ml.), and the reaction mixture was left in the refrigerator overnight. The clear solution obtained was dialyzed against water for five days at room temperature. The solution left in the cellophane bag was concentrated *in vacuo* and dried over sulfuric acid; yield 0.87 g.

Both the non-dialyzable and the dialyzable material were hydrolyzed in 10 *N* hydrochloric acid at 110° for 24 hours, and the hydrolysates were chromatographed on paper, using 1-butanol–pyridine–glacial acetic acid–water (15:10:3:12) as the mobile phase. The hydrolysate of the non-dialyzable material contained lysine and sarcosine, while the hydrolysate of the material that passed through the cellophane membrane was composed of sarcosine exclusively, as revealed by spraying with ninhydrin.

The amount of lysine (23 mg.) found per 100 mg. of the multi-polysarcosylpolylysine, as determined by a quantitative chromatographic analysis[12] of the hydrolysate, corresponds to a molar ratio of 7.1 to 1 of sarcosine to lysine, *i.e.*, an average molecular weight of $(7.1 \times 71) + (1 \times 128) = 632$, for each side chain (including the lysine residue) of the multichain polysarcosine. An average molecular weight of 585, for each chain of the branched polymer, was calculated from end-group analysis.[2] The free imino groups were determined by titration in glacial acetic acid with perchloric acid in glacial acetic acid, using crystal violet as indicator[2] (equiv. wt., 850). The carboxyl groups were determined by titration in ethanol with sodium methoxide in benzene–methanol, using thymol blue as indicator[2] (equiv. wt., 1860); Van Slyke analysis, amino N, 0.0.

The hygroscopic multi-polysarcosyl-poly-L-lysine (*b* 20, *p* 7) is soluble in water, in ethanol and glacial acetic acid, and is insoluble in dimethylformamide.

Multi-poly-L-lysyl-poly-L-lysine Hydrobromide.—An ice-cooled solution of ε,N-carbobenzoxy-α,N-carboxy-L-lysine anhydride[16] (9.0 g.) in dioxane (130 ml.) was mixed with an ice-cooled solution of poly-L-lysine hydrobromide[5] (DP 30) in *M*/75 phosphate buffer *p*H 7 (120 ml.), and the reaction mixture was left in the refrigerator overnight. The precipitate formed was filtered, and washed thoroughly with 0.2 *N* hydrochloric acid, 0.2 *N* sodium hydroxide and with water. The dried material was treated with a 33% solution of hydrogen bromide in glacial acetic acid[20] (20 ml.). The precipitation of the decarbobenzoxylated product was completed after 30 minutes by the addition of anhydrous ether (100 ml.). The precipitate was dissolved in water (50 ml.) and then dialyzed against 0.001 *N* aqueous hydrobromic acid. The solution left in the cellophane bag was concentrated *in vacuo* and dried over sulfuric acid and potassium hydroxide; yield 1.84 g. The dried polymer gave: amino N, 6.5 (Van Slyke); Br, 37.0 (Volhard).

Multi-polylysyl-polylysine was desaminated analogously to the desamination of multi-poly-DL-alanyl-poly-L-lysine.

(24) A. L. Levy, *Nature*, **174**, 126 (1954).

The product formed, which precipitated out from the reaction mixture, was filtered, washed with water, and subjected to hydrolysis and chromatographic analysis as in the case of multipolyalanyl-polylysine. Four spots were revealed after spraying with ninhydrin: one corresponding to lysine and three with R_x values identical with those given previously for a hydrolysate of the desamination product of a linear polylysine. The amount of lysine (4.5 mg.) found per 100 mg. of the original multi-polylysylpolylysine hydrobromide, as determined by quantitative chromatographic analysis, showed that on the average 15 lysine residues are attached to each ϵ-amino group of the polylysine (DP 30) used as multifunctional initiator. The ninhydrin colorimetry[12] of the lysine and of the combined amino acids resulting from the desaminated lysine residues gave an optical density ratio of 1 to 14. As the color developed by lysine with ninhydrin is 1.1 times that developed by leucine and most other α-amino acids,[12] it was assumed that the molar absorption of the three unknown spots is similar to that of leucine. The above mentioned ratio between optical densities corresponds thus to an average side chain length of 15.6 lysine residues (the lysine residue serving as initiator excluded).

A linear poly-L-lysine hydrobromide, obtained under conditions identical with those given for the preparation of multi-poly-L-lysyl-poly-L-lysine hydrobromide, but in the absence of the multifunctional initiator, yielded a material 98% of which passed through the cellophane membrane.

Multi-poly-L-lysyl-poly-L-lysine hydrobromide (b 30, p 15) is soluble in water. The free multichain base may be precipitated from the aqueous solution of the hydrobromide by the addition of sodium hydroxide.

Multi-poly-L-lysyl-poly-L-lysine hydrobromide (b 30, p 15) (26 mg.) was incubated with crystalline trypsin (0.3 mg.) in 0.1 N phosphate buffer, pH 7.6 (1 ml.) for 48 hours at 25°. Chromatography of the reaction mixture showed that multi-poly-L-lysyl-poly-L-lysine was digested quantitatively to lysyllysine and traces of lysine.[11]

The hydrobromide of multi-poly-L-lysyl-poly-DL-ornithine was prepared similarly to that of multi-poly-L-lysyl-poly-L-lysine, but using poly-DL-ornithine[13] (DP 36) as polyfunctional initiator. Both the dialysable and the nondialysable products were hydrolyzed as previously, and the hydrolysates were analyzed by paper electrophoresis. The electrophoretic runs were carried out in $M/20$ disodium phosphate on a Whatman No. 1 filter paper at a potential gradient of 10 volt/cm., at 25°. After three hours the amino acid spots were revealed with ninhydrin. Under these conditions ornithine stayed at the origin, while lysine migrated 3.8 cm. toward the cathode. The hydrolysate of the dialyzable material was found to contain lysine exclusively, while that of the non-dialyzable material was found to contain lysine and ornithine in a molar ratio of 3 to 1, as determined colorimetrically.

Multi-polylysyl-polyornithine was subjected to desamination followed by hydrolysis in a manner described in the desamination experiment of multi-poly-DL-alanyl-poly-L-lysine. The hydrolysate was analyzed electrophoretically and found to contain ornithine but no lysine.

Multi-poly-L-lysyl-poly-DL-ornithine hydrobromide (b 36, p 3) (24 mg.) was incubated with crystalline trypsin (3 mg.) in 0.1 M phosphate buffer, pH 7.6 (1 ml.) for 48 hours at 25°. The reaction mixture was chromatographed[11] as a band, and showed after spraying with ninhydrin, a band of lysyllysine, a band of lysine, and a band at the origin. The latter was cut out, eluted and hydrolyzed with 6 N hydrochloric acid at 110° for 24 hours. The hydrolysate was found to contain lysine and ornithine in an approximate molar ratio of 1 to 2, as determined by ninhydrin colorimetry[12] after paper electrophoresis.

Multi-poly-L-tyrosyl-poly-L-lysine.—An ice-cooled solution of O-carbobenzoxy-N-carboxy-L-tyrosine anhydride[17] (6.0 g.) in dioxane (60 ml.) was mixed with an ice-cooled solution of poly-L-lysine hydrobromide,[5] DP 200 (150 mg.) in $M/75$ phosphate buffer, pH 7 (100 ml.), and the reaction mixture was left in the refrigerator overnight. The precipitate formed was filtered and washed thoroughly with 0.2 N hydrochloric acid, 0.2 N sodium hydroxide and with water. The dried material was treated with a 33% solution of hydrogen bromide in glacial acetic acid[20] (20 ml.) and the decarbobenzoxylated product was precipitated after 30 minutes with anhydrous ether, dried, dissolved in ethanolic sodium hydroxide and dialyzed against ethanol for six days. The solution left in the cellophane bag was concentrated *in vacuo* and dried. The transparent film obtained was triturated with 0.1 N hydrochloric acid, washed with water and dried; yield 1.2 g.

The multichain polytyrosine was hydrolyzed as described previously. A quantitative chromatographic analysis of the hydrolysate, using 1-butanol–glacial acetic acid–water (4:1:5) as the mobile phase, gave an approximate molar ratio of tyrosine to lysine, 8 to 1. The intact multichain polytyrosine gave amino N, 0.88 (Van Slyke).

Multi-poly-L-tyrosyl-poly-L-lysine (b 200, p 8) is soluble in aqueous or alcoholic alkali, partially soluble in ethanol, pyridine and hot dimethylformamide, and is insoluble in water, dioxane and ether.

Multi-poly-L-glutamyl-poly-L-lysine. (a) **In Phosphate Buffer, pH 7.**—An ice-cooled solution of γ-benzyl-N-carboxy-L-glutamate anhydride[18] (14.0 g.) in dioxane (140 ml.) was mixed with an ice-cooled solution of poly-L-lysine hydrobromide,[5] DP 200 (310 mg.) in $M/75$ phosphate buffer, pH 7 (140 ml.), and the reaction mixture was left in the refrigerator overnight. The precipitate formed was filtered, washed with water and dried *in vacuo*. The dried polymer was dissolved in glacial acetic acid (70 ml.), the solution heated to 70° and dry hydrogen bromide passed in for one hour.[20] The debenzylated product was precipitated with anhydrous ether (300 ml.), filtered, washed with ether, dried and dissolved in water at pH 8 (sodium hydroxide). The solution was dialyzed for 24 hours against 0.002 N sodium hydroxide and then for four days against water. The solution left in the dialysis bag was concentrated *in vacuo* and dried over phosphorus pentoxide. The transparent film left was triturated with glacial acetic acid (20 ml.) and anhydrous ether (150 ml.), and the white precipitate formed washed several times with ether and dried *in vacuo;* yield 1.0 g.

The multichain polyglutamic acid was hydrolyzed and the hydrolysate analyzed chromatographically analogously to the multichain polytyrosine. A molar ratio of glutamic acid to lysine, 5.7 to 1, was obtained. The intact multichain polyglutamic acid gave amino N, 1.67 (Van Slyke) and Na, 3.1, as determined with a flame spectrophotometer (model Beckman G). The sodium content corresponds approximately to one sodium per five glutamic acid residues.

The electrophoretic mobility of multi-poly-L-glutamyl-poly-L-lysine (b 200, p 6) was compared with that of linear poly-L-glutamic acid[13] (DP 20), of linear poly-L-lysine (DP 200), as well as with that of a mixture of linear polyglutamic acid (DP 20) and linear polylysine (DP 200) in a ratio of six glutamic acid residues to one lysine residue. The measurements were carried out on a Whatman No. 1 filter paper in $M/20$ disodium phosphate, at a potential gradient of 10 volt/cm., at 25°. After three hours the polymers were revealed with ninhydrin. The linear polyglutamic acid migrated as an elongated spot for a distance of 8.8 cm. toward the anode. The linear polylysine spread into an elongated spot of 3.2 cm. length toward the cathode. The mixture of the two linear polymers gave a circular spot at the origin, probably representing the interaction product of polylysine and polyglutamic acid, and an elongated linear polyglutamic acid spot that migrated for a distance of 9.0 cm. toward the anode. The multi-polyglutamyl-polylysine migrated 6.5 cm. toward the anode as one spot.

The multichain polyglutamic acid gives a positive ninhydrin reaction and a strongly positive biuret test. With cupric ions it gives a complex insoluble at pH values higher than 4.

(b) **In 97% Dioxane.**—An aqueous solution (10 ml.) of free polylysine, prepared by the neutralization of poly-L-lysine hydrobromide, DP 200 (610 mg.), with triethylamine (0.5 ml.), was added dropwise to a solution of γ-benzyl-N-carboxy-L-glutamate anhydride[18] (18.0 g.) in anhydrous dioxane (300 ml.) with vigorous stirring. The multichain polymer obtained was precipitated, after 24 hours, from the cloudy reaction mixture with water. The precipitate was left in the refrigerator overnight, filtered, washed with water, and dried *in vacuo* over sulfuric acid and potassium hydroxide; yield 14.0 g.

The multi-poly-γ-benzyl-glutamyl-poly-L-lysine preparation is soluble in pyridine, dimethylformamide and hot glacial acetic acid from which it precipitates on cooling. It is partially soluble in chloroform and nitromethane, and insoluble in dioxane, acetone and carbon tetrachloride.

The glutamate polymer (10 g.) was debenzylated as described above (*cf.* section a); yield 5.8 g. The debenzylated product was dissolved in an excess of sodium hydroxide

and back-titrated with hydrochloric acid, using brom thymol blue as indicator: equiv. wt., 125.

The debenzylated polymer was dialyzed and treated as in section a; yield 4.6 g. The multichain polyglutamic acid was hydrolyzed and the hydrolysate analyzed chromatographically analogously to the multichain polytyrosine. A molar ratio of glutamic acid to lysine, 16 to 1, was obtained. The intact multichain polyglutamic acid gave amino N, 0.60 (Van Slyke), and Na, 2.9, determined with a flame spectrophotometer. The multichain polyglutamic acid was desaminated analogously to the desamination of multichain poly-DL-alanine, and the product hydrolyzed as usual. The hydrolysate was analyzed chromatographically, using 1-butanol–glacial acetic acid–water (4:1:5) as the mobile phase. The chromatogram contained spots of lysine and glutamic acid, but no spots corresponding to the products appearing in the hydrolysate of desaminated polylysine.

Poly-L-lysine, Free Base.—A solution of poly-L-lysine hydrobromide, DP 18 (78 mg.), in water (5 ml.) was passed through a column of Amberlite IRA 400, and the fraction giving a negative Volhard and a positive ninhydrin test (20 ml.) was collected and dried *in vacuo*; yield, 45 mg. of a white powder.

Anal. Calcd. for poly-L-lysine (DP 18): neut. equiv., 128. Found: neut. equiv., 134, determined by titration in glacial acetic acid with perchloric acid in glacial acetic acid, using thymol blue as indicator.

The polylysine free base (DP 18) obtained could not be redissolved in water. It is soluble in glacial acetic acid, in phenol and in aqueous acids. It is insoluble in ether, dioxane, methanol, pyridine, triethylamine, tributylamine and boiling dimethylformamide. It may be precipitated from its solution in glacial acetic acid by dioxane.

Physical Measurements.—The sedimentation measurements were carried out in a Spinco ultracentrifuge Model E with a Klett optical system. The sedimentation constant was calculated as an average of the values $S = 2(x_2 - x_1)/(x_2 + x_1)\omega^2(t_2 - t_1)$, where x_1 and x_2 are the distances from the axis of rotation to the boundary at the times t_1 and t_2, respectively, and ω is the angular velocity. Final corrections were made in the customary manner to a solvent with the density and viscosity of water at 20°. For this purpose a partial specific volume of 0.72 was employed.

Diffusion measurements were carried out in a Claesson diffusion cell,[25] at 20°. A Tiselius–Longsworth apparatus[26] with a schlieren-scanning optical system was used. In the measurements made the diffusion curves were found to be practically normal. The average diffusion coefficients were derived using the formula $D = \sigma/2t$, where σ denotes the standard deviation and t the time of diffusion. The standard deviation was calculated from the first and second moments measured with an Amsler Integrator.

The refractive index of multi-poly-DL-alanyl-poly-L-lysine (*b* 34, *p* 22) in glycine buffer, *p*H 9.1 and ionic strength 0.1, at 20°, was measured by means of a dipping refractometer compensated to sodium light. The refractive index increment, $dn/dw = 0.182$, was calculated from the slope of linear relation between refractive index and concentration found experimentally (w is the ratio between the weight of solute and the weight of solution).

Viscosity measurements were carried out in an Ostwald viscosimeter at 20°.

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CONTRIBUTION FROM THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Chromatography of Proteins. I. Cellulose Ion-exchange Adsorbents

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Ion-exchange adsorbents have been prepared from cellulose under conditions such that physical properties suitable for column chromatography are maintained. These adsorbents possess high capacity for the adsorption of proteins, yet permit elution under mild conditions. Titration curves are presented.

The remarkable success of chromatography in the separation of amino acids, lipids, sugars and many other substances has been an important factor in recent advances in biochemistry. The application of chromatography to the separation of proteins, however, has not met with the general success obtained with smaller, simpler molecules. The large size of the protein molecule prevents its penetration into the adsorbent particle, therefore only the adsorbing sites located on the exterior can be utilized. An enormous surface, as provided by very fine division, then becomes necessary for the attainment of reasonably high adsorption capacity. The instability of the protein molecule is also a factor, not only because of the severe limitations it imposes upon the choice of solvents, but also because profound configurational changes may accompany the adsorptive and desorptive processes if binding sites are sufficiently numerous and strong to compete with the multitude of weak bonds which maintain the native configuration of the protein. Even where this does not occur, too high a density of binding sites may result in adsorption so firm that the conditions required for simultaneous dissociation of all the adsorptive bonds are destructive to the integrity of the molecule.

However, progress has been made in recent years, particularly in the field of ion-exchange chromatography. Relatively stable proteins of rather low molecular weight and high isoelectric point have been chromatographed on columns of finely divided Amberlite IRC-50, a weak cation exchanger.[1] The chromatography of hemoglobin, a neutral molecule of more typical size, has been demonstrated on the same resin, but the conditions required were such as to preclude ion exchange as the adsorption mechanism.[2] Very recently the separation of four kinds of human carbon monoxide hemoglobin with IRC-50 has been reported.[3] Early work in this Laboratory achieved the frontal analysis of artificial mixtures of typical proteins on the strong cation exchanger, Dowex-50, but the capacity was very low.[4] The same resin has been used for the chroma-

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