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The Terminal Groups of Poly-α-amino Acids

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The terminal groups of various poly-α-amino acids, prepared by polymerization of N-carboxy-α-amino acid anhydrides, were investigated. The formation of ureido end groups due to a termination reaction 3 was demonstrated by the isolation of hydantoin-3-acetic acid derivatives from the hydrolysates of purified poly-α-amino acids. A method for the quantitative colorimetric determination of hydantoin-3-acetic acids, and a procedure for the anhydrous titration of acidic and basic end groups in poly-α-amino acids, are described. The quantitative analysis of all the end groups was shown to be consistent with the postulated termination mechanism. The evaluation of degrees of polymerization from end group analyses is discussed.

Many poly-α-amino acids have been synthesized recently by polymerization of N-carboxy-α-amino acid anhydrides.[1] A mechanism of polymerization consisting of initiation 1 and propagation 2 reactions, was assumed by various authors.[2-4] Accordingly, when water, amines or alcohols (X = OH, R_2N, OR) are used as initiators, every polymeric chain should contain a terminal amino group. Water-initiated polymers should contain, in addition, one carboxyl group per chain, while the other initiators should give rise to amide or ester groups instead.

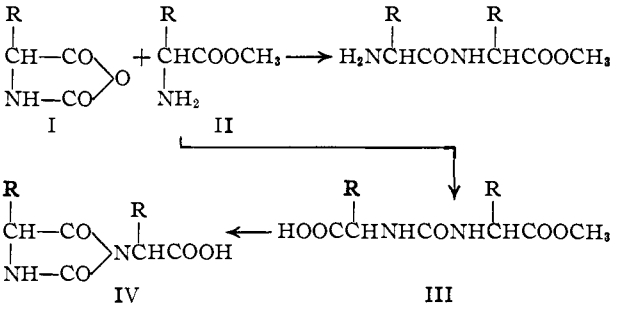
$$XH + \overline{OCONHCHRCO} \longrightarrow XCOCHRNH_2 + CO_2 \quad (1)$$

$$X(COCHRNH)_nH + \overline{OCONHCHRCO} \longrightarrow X(COCHRNH)_{n+1}H + CO_2 \quad (2)$$

In a preliminary communication[5] evidence was presented, based on a study of the terminal groups of the polymerization products, for the occurrence of a termination reaction 3, in addition to reactions 1 and 2.

$$X\cdot(COCHRNH)_mH + \begin{array}{l} CO{-}CHR \\ O \\ CO{-}NH \end{array} \longrightarrow X\cdot(COCHRNH)_m\cdot CONHCHRCOOH \quad (3)$$

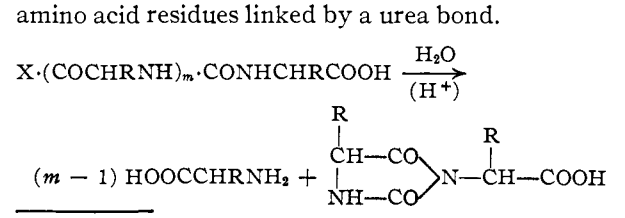
In the termination reaction, the amino group of a growing peptide chain reacts with carbon 2 of the N-carboxy-α-amino acid anhydride to give a urea derivative with a free carboxyl group. Such a reaction seemed possible because N-carboxy-α-amino acid anhydrides (2,5-oxazolidinediones) react with amines and alcohols through the C-2 carbonyl as well as through the C-5 carbonyl group. Thus methanolic sodium methoxide reacts with N-carboxy-α-amino acid anhydrides to yield N-carbomethoxyamino acids in addition to amino acid esters.[6] Similarly it was observed that on treating N-carboxy-L-phenylalanine anhydride (I, R = $C_6H_5CH_2$–) with an excess of L-phenylalanine methyl ester (II), a small amount of carbonyl-bisphenylalanine methyl ester (III) was formed, whose presence was proved by the isolation of L,L-α,5-dibenzylhydantoin-3-acetic acid[7] (IV) after acid hydrolysis.



It was shown recently that isatoic anhydride yields with primary and secondary amines not only the corresponding anthranilamides, but also *o*-uramidobenzoic acids.[8]

Polymers isolated from amine-initiated polymerization reaction mixtures and carefully freed from unreacted anhydride, contained, as expected from reaction 3, acid groups titratable by sodium methoxide in various anhydrous solvents. In water-initiated preparations or in bulk polymers the number of carboxyl groups always exceeded that of terminal amino groups as determined either by Van Slyke analysis or, more conveniently, by titration with perchloric acid in anhydrous solvents (*cf.* Table I).

The occurrence of the termination reaction was proven conclusively by the isolation of hydantoin-3-acetic acid derivatives from acid hydrolysates[7] of various poly-α-amino acids prepared under different conditions. These hydantoin-3-acetic acid derivatives are formed from the two terminal amino acid residues linked by a urea bond.



(1) A review of the literature on the subject up to 1950 is given by E. Katchalski, *Advances in Protein Chem.*, **6**, 123 (1951). See also, W. E. Hanby, S. G. Waley and J. Watson, *J. Chem. Soc.*, 3009, 3239 (1950); D. Coleman, *ibid.*, 2294 (1951); A. Berger and E. Katchalski, THIS JOURNAL, **73**, 4084 (1951); M. Green and M. A. Stahmann, *J. Biol. Chem.*, **197**, 771 (1952); M. Frankel, M. Breuer and S. Cordova, *Experientia*, **8**, 299 (1952); K. Schlögl, F. Wessely and G. Korger, *Monatsh.*, **83**, 845 (1952); M. Frankel, M. Harnik, Y. Levin and Y. Knobler, THIS JOURNAL, **75**, 78 (1953); H. Tani, H. Yuki, S. Sakakibara and T. Taki, *ibid.*, **75**, 3042 (1953); E. Katchalski and M. Sela, *ibid.*, **75**, 5284 (1953); M. Sela and E. Katchalski, *ibid.*, **76**, 129 (1954); A. Patchornik, M. Sela and E. Katchalski, *ibid.*, **76**, 299 (1954).

(2) R. B. Woodward and C. H. Schramm, *ibid.*, **69**, 1551 (1947).

(3) S. G. Waley and J. Watson, *Proc. Roy. Soc. (London)*, **A199**, 499 (1949).

(4) L. Gold, *J. Chem. Phys.*, **21**, 1190 (1953).

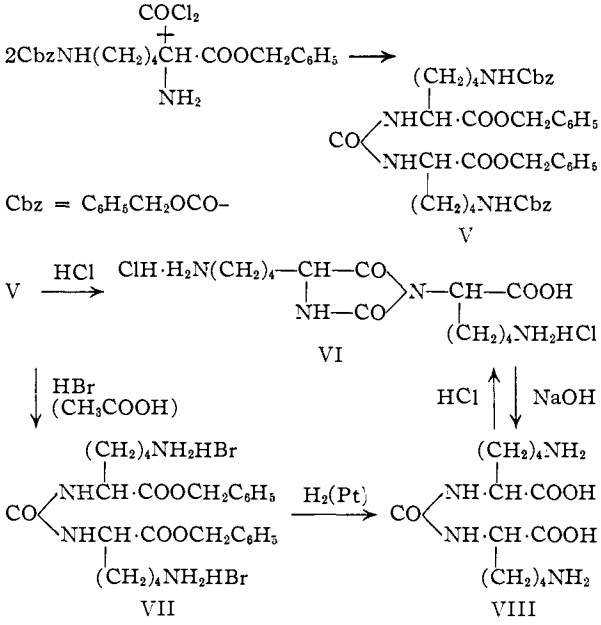
(5) M. Sela and A. Berger, THIS JOURNAL, **75**, 6350 (1953).

(6) A. Berger, M. Sela and E. Katchalski, *Anal. Chem.*, **25**, 1554 (1953).

(7) F. Wessely and J. Mayer, *Monatsh.*, **50**, 439 (1928).

(8) R. P. Staiger and E. C. Wagner, *J. Org. Chem.*, **18**, 1427 (1953).

Thus L,L-α,5-dibenzylhydantoin-3-acetic acid[7] (IV) was isolated from an acid hydrolysate of poly-L-phenylalanine, while both the racemic forms[9] of IV were obtained from the hydrolysate of poly-DL-phenylalanine. Poly-DL-alanine yielded a mixture of the two racemic forms of α,5-dimethylhydantoin-3-acetic acid.[10] The experimental procedure applied in the above cases, namely, extraction of the hydantoin-3-acetic acid derivatives by means of organic solvents, could not be used in the case of poly-L-lysine. In order to detect the presence of L,L-α,5-di-(4-aminobutyl)-hydantoin-3-acetic acid (VI) in the hydrolysate of poly-L-lysine, this compound was synthesized for comparison.



ϵ,N-Carbobenzoxy-L-lysine benzyl ester reacted with phosgene to yield carbonyl-bis-(ϵ,N-carbobenzoxy-L-lysine benzyl ester) (V) which was converted into the hydantoin derivative VI either directly by treatment with acetic acid–hydrochloric acid or *via* the carbonyl-bis-lysine dibenzyl ester (VII) and carbonyl-bis-lysine (VIII).

The hydantoin derivative of lysine (VI), analogously to the corresponding derivatives of phenylalanine and alanine,[11] produces a red color on treatment with picric acid in alkaline solution. On acidification the color becomes orange-red. These acid solutions have a characteristic absorption band in the range from 4500 to 5500 Å. with a maximum at 4770 Å. (extinction 1330 per mole of α,5-dibenzylhydantoin-3-acetic acid and 1290 per mole of α,5-di-(4-aminobutyl)-hydantoin-3-acetic acid present originally).

The color produced with the hydantoin derivative of phenylalanine (IV) is extracted from the acid solution by ethyl acetate, while in the hydantoin derivative of lysine (VI) the color remains in the aqueous layer. The color formed in all the above cases is probably the result of a complex formed between hydantoin derivatives and picric acid, similar to the complex of creatinine with picric acid.[12]

On paper chromatograms the lysine hydantoin derivative VI gave a single spot which could be revealed by its fluorescence in ultraviolet light, by spraying with ninhydrin, or, at higher concentrations, by spraying with alkaline picric acid solution. The R_f in *n*-butyl alcohol–acetic acid–pyridine–water (15:3:10:12)[13] is identical with that of lysine.

Hydrolysates (hydrochloric acid–acetic acid mixture) of various poly-L-lysine preparations gave with picric acid a red compound which could not be extracted from acid solution and whose spectrum in the range from 4500 to 5500 Å. was identical with that of VI. Chromatography of the hydrolysates showed in ultraviolet light several bands corresponding to lysine and lysine peptides.[14] When these bands were cut out and eluted, only the band corresponding to VI and lysine gave a positive picric acid reaction.

Based on the picric acid color reaction of the hydantoin-3-acetic acid derivatives, a method for their quantitative determination was developed. The amount of the colored compound produced in alkaline solution was determined, after acidification with acetic acid, at 5000 Å., where the absorption of excess picric acid is negligible. The colored solutions obtained from different hydantoin-3-acetic acid derivatives (*e.g.*, of phenylalanine, alanine, lysine) showed extinctions close to 930 per mole of hydantoin-3-acetic acid derivative present originally.

Generally, poly-α-amino acids prepared from N-carboxy-α-amino acid anhydrides may contain the following four types of terminal groups: (1) substituted carboxyl groups (such as amide, ester etc.) arising from the action of any initiator other than water; (2) carboxylic groups arising from initiation by water; (3) amino groups formed by the propagation reaction (unterminated chains); (4) carboxylic groups arising from the termination reaction (these are adjacent to the urea groups). The relation between the concentrations of the four terminal groups in a given polymer is expressed by

$$C + (A - U) = B + U \qquad (4)$$

where C (for Catalyst) is the number of moles of substituted carboxyl groups (1), per one mole of amino acid residue; A (for Acid) the total number of carboxylic terminal groups, (2) and (4), per one mole of amino acid residue; B (for Base) is the number of moles of terminal amino groups, (3), per one mole of amino acid residue; and U (for Urea) is the number of moles of carboxylic groups adjacent to urea groups, (4), per one mole of amino acid residue. The four quantities A, B, C and U, can, under favorable conditions, be determined independently. Experimental values are given in Table I.

A values were obtained from the results of titrations in anhydrous dimethylformamide with sodium methoxide solution, using thymol blue as indica-

(9) F. Wessely and M. John, *Z. physiol. Chem.*, **170**, 167 (1927).

(10) Ch. Gränacher and G. Wolf, *Helv. Chim. Acta*, **11**, 172 (1928).

(11) Ch. Gränacher and H. Landolt, *ibid.*, **10**, 799 (1927).

(12) W. K. Anslow and H. King, *J. Chem. Soc.*, 1210 (1929).

(13) S. G. Waley and J. Watson, *Biochem. J.*, **55**, 328 (1953).

(14) S. G. Waley and J. Watson, *J. Chem. Soc.*, 475 (1953).

TABLE I

END GROUP DETERMINATIONS OF POLY-α-AMINO ACIDS

A = number of moles of terminal carboxyl groups per mole of amino acid residue (titration); B = number of moles of terminal amino groups per mole of amino acid residue (titration); B' = number of moles of terminal amino groups per mole of amino acid residue (Van Slyke); C = number of moles of amine-initiator groups per mole of amino acid residue (determined spectrophotometrically when L-tyrosine ethyl ester was used); C = zero, in water-initiated or bulk polymerization; values given in brackets were not determined, but calculated from $C = (B + 2U - A)$; U = number of moles of carboxyl groups adjacent to urea groupings per mole of amino acid residue (determined spectrophotometrically as picric acid complex of the hydantoin-3-acetic acid derivative, unless specified otherwise).

	Poly.	A	B	B'	C	U	$C + A - U$	$B + U$	DP[a]	$1/B'$ or $1/B$	$C/(C + A - U$
1	DL-Phenylalanine[b]	0.033	0.011	0.010	0	(0.0085[c])	(0.0245)	(0.0195)	45	100	
2	L-Phenylalanine[d]	.000	.024		[0.028]	.002		.026	39	42	1.08
3	L-Phenylalanine[e]	.034	.000		[.028]	.031		.031	32	>1000	0.90
4	L-Phenylalanine[f]	.056	.010		0	(.018[c])	(.038)	(.028)	30	100	
5	L-Phenylalanine[g]	.077	.000		0	(.013[c])	(.064)	(.013)	26	>1000	
6	DL-Ornithine[h]	.048[i]	.017[i]	.014[i]	0				31	71	
7	L-Aspartic acid[j,k]	.048	.073		0.077	.022	.103	.095	10	14	0.75
8	L-Lysine[l,m]	.118[i]	.059[i]	.059[i]	0	.031	.087	.090	11	17	
9	L-Lysine[b,l]	.091[i]	.020[i]	.019[i]	0				18	53	
10	L-Lysine[b,l]	.072[i]	.016[i]	.016[i]	0				23	63	
11	L-Lysine[b,l]	.056[i]	.016[i]	.012[i]	0				28	83	
12	L-Lysine[l,n]	.120[i]	.052[i]		0	.031	.089	.083	12	19	
13	L-Lysine[b,l]	.083[i]	.019[i]		0	.037	.046	.056	20	53	
14	L-Lysine[l,n]	.030[i]	.020[i]		[0.050]	.030		.050	20	50	1.00
15	L-Lysine[k,l,o]	.016[i]	.012[i]		.029	.018	.027	.030	35	83	1.08
16	L-Lysine[k,l,o,p]	.112[i]	.048[i]		.045	.048	.109	.096	10	21	0.41
17	L-Lysine[k,l,o,p]	.061[i]	.044[i]		.025	.023	.063	.067	15	23	0.40
18	DL-Alanine[q,r]	.050		.003	0	(.016[c])	(.034)	(.019)	38	333	
19	DL-Alanine[q,r]	.039		.021	0	.012	.027	.033	33	48	
20	DL-Alanine[k,p,r,s]	.058		.022	0.016	.022	.052	.044	21	45	0.31
21	DL-Alanine[k,p,r,t]	.055		.030	.016	.019	.052	.049	20	33	.31
22	DL-Alanine[p,u]	.028		.028	.017	.011	.034	.039	27	36	.50
23	L-Proline[v]	.029		.000					35	>1000	

[a] Calculated from $2/(A + B + C)$, or from $1/(B + U)$, where C not available. [b] Bulk polymerization; purified by precipitation with water from dimethylformamide solution and washed with ethyl acetate. [c] Calculated from the weight of the hydantoin-3-acetic acid derivative isolated, and therefore not quantitative. [d] Polymerization in dioxane at 20°, initiated by diethylamine. [e] Polymerization in boiling dioxane, initiated by diethylamine. [f] Bulk polymerization; washed with dimethylformamide. [g] Polymerization in dioxane at 20°, initiated by water. [h] Prepared by decarbobenzoxylation with hydrogen bromide[16] from poly-δ-carbobenzoxy-DL-ornithine[15] synthesized in bulk and washed with ethyl acetate. [i] Determined before decarbobenzoxylation. [j] Prepared by debenzylation with hydrogen bromide[16] from polybenzyl-L-aspartate[17] synthesized in boiling dioxane. [k] Initiated by L-tyrosine ethyl ester. [l] Prepared by decarbobenzoxylation with hydrogen bromide,[16] from poly-ε-carbobenzoxy-L-lysine.[18] [m] Polymerization in boiling benzene, initiated by water; purified by precipitation with water from dimethylformamide solution and washed with ethyl acetate. [n] Polymerization in dimethylformamide at 20°, initiated by diethylamine; precipitated by water and washed with ethyl acetate. [o] Polymerization in dioxane at 20°; washed with ethyl acetate; purified by precipitation with water from dimethylformamide solution. [p] Prepared from N-carboxyanhydrides kept in desiccator over sulfuric acid and potassium hydroxide for several weeks before polymerization. [q] Bulk polymerization. [r] Purified by precipitation with acetone from concentrated aqueous solution. [s] Polymerization in boiling dioxane. [t] Polymerization in dioxane at 20°. [u] Sample 21, dialyzed for 12 hours against water. [v] Prepared in solution according to Berger, *et al.*[19]

tor.[20] End-points were sharp, and reproducible results could be obtained even when the polymer did not dissolve and was present as a suspension of swollen particles. B values were determined by titration in glacial acetic acid with perchloric acid in glacial acetic acid, using crystal violet as indicator, or in anhydrous dimethylformamide with perchloric acid in dioxane, using thymol blue as indicator. These values were generally in good agreement with those obtained by Van Slyke amino nitrogen analysis (B' in Table I). The titration was preferred when the polymer was water insoluble, while in the case of substances soluble in water, but insoluble in anhydrous solvents (*e.g.*, poly-DL-alanine), the Van Slyke method seemed more reliable. The U values were obtained from the colorimetric determination of the hydantoin-3-acetic acid derivatives, assuming they were formed in quantitative yield on acid hydrolysis. In the earlier stages of this work the hydantoin-3-acetic acid derivatives were isolated and weighed. U values obtained in this way are given in brackets and are probably low. C values were easily estimated when tyrosine ethyl ester was used as initiator of polymerization, the amount of tyrosine being determined spectrophotometrically.[21] C was

(15) E. Katchalski and P. Spitnik, THIS JOURNAL, **73**, 3992 (1951).

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

(17) A. Berger and E. Katchalski, THIS JOURNAL, **73**, 4084 (1951).

(18) E. Katchalski, I. Grossfeld and M. Frankel, *ibid.*, **70**, 2094 (1948).

(19) A. Berger, J. Kurtz and E. Katchalski, *ibid.*, **76**, 5552 (1954).

(20) D. Coleman and A. C. Farthing (*J. Chem. Soc.*, 3218 (1950)) report the determination of acidic end-groups in a L-leucine-DL-phenylalanine copolymer by means of absorption of methylene blue. J. H. Fessler and A. G. Ogston (*Trans. Faraday Soc.*, **47**, 667 (1951)) titrated polysarcosine in aqueous solution, but stated that their values give no more than an indication of the number of acid groups present, as the end-point was arbitrarily fixed.

(21) Other initiators such as ammonia and volatile amines can be determined with accuracy as amide nitrogen (R. R. Becker and M. A. Stahmann, THIS JOURNAL, **74**, 38 (1952)).

assumed to be zero in the case of water-initiated or bulk polymerizations.

The above considerations concerning the various types of end-groups are supported by the reasonable agreement between the values of $C + A - U$ and $B + U$ (*cf.* equation 4). The main error lies probably in the U values, where, in addition to the low accuracy inherent in the colorimetric determination, there is some uncertainty as to the quantitative formation of the hydantoin derivatives during the partial hydrolysis.

The values of A, B and C permit the calculation of the number average degree of polymerization, DP

$$\mathrm{DP} = \frac{2}{A + B + C} \qquad (5)$$

In cases of water-initiated or bulk polymerization this reduces to $2/(A + B)$. Values of DP calculated from formula 5 are given in Table I. They are generally lower than those usually derived from amino end-group determinations only, where the DP was assumed to be equal to $1/B$. In principle, DP can be calculated also from $1/(C + A - U)$ or $1/(B + U)$. The last expression was used for the calculation of DP in cases where C values were not determined.

The values in column $C/(C + A - U)$ give the fraction of polymeric chains formed by amine-initiations, in cases where amines were used as starters. In the cases (samples 16, 17, 20, 21, 22) where these values are significantly lower than unity, a high proportion of water-initiated chains was probably present.

The termination reaction formulated in equation 3 explains the recurrent observation[1] that in most polymerizations of N-carboxy-α-amino acid anhydrides the amount of carbon dioxide evolved is less than one equivalent per mole of anhydride. Breitenbach and Allinger,[22] for example, stated in a recent kinetic study of the polymerization of N-carboxy-DL-phenylalanine anhydride that the amount of carbon dioxide evolved was always significantly lower than the calculated, sometimes as low as 88% of the theoretical. In attempting to explain this discrepancy they considered the possibility of formation of urea groupings, through a mechanism different from the one proposed by us. A similar view was expressed by Heyns and Brockmann.[23] Sluyterman and Labruyère[24] confirm our findings[5] concerning the excess of carboxyl groups over amino groups in water-initiated poly-α-amino acids. These authors suggest that the polypeptides contain terminal hydantoin rings formed by the splitting off of water from the intermediate N-carboxypeptides. The excess of the carboxyl groups thus would result only from the conversion of amine groups into hydantoin rings. The positive picric acid test of their polyglycine preparations, offered as evidence for this view, appears inconclusive, as this color reaction, although negative for carbonyl-bis-amino acids, is positive for carbonyl-bis-amino acid esters[11] and amides (*e.g.*, carbonyl-bis-alanine diethyl ester, carbonyl-bis-DL-alanine dibutylamide), as well as for carbonyl-(glycine)-(glycylglycine).[25] We believe that this mechanism cannot be correct, since it does not account for the carboxyl groups found in amine-initiated polymers, nor can it explain the absence of imino end-groups in preparations of poly-L-proline,[19] where the formation of terminal hydantoin rings is impossible. In order to investigate the possibility of the formation of hydantoin rings from N-carboxypeptides during polymerization, we prepared the carbamate salt[26] of triglycine methyl ester[27] and heated it to 110° for several hours. Although in this experiment 50% of the tripeptide was present in the form of N-carboxypeptide, no hydantoin formation occurred, as is evident from the negative picric acid reaction of the resulting substance.

It is believed that a thorough investigation of the kinetics of the termination reaction may lead to better control of the molecular weights of poly-α-amino acid preparations. A theoretical treatment of the kinetics of the polymerization of N-carboxy-α-amino acid anhydrides, taking into account the termination reaction suggested here, is given by E. Katchalski, Shalitin and Gehatia.[28]

Experimental

All melting points are uncorrected. Amino acids from Nutritional Biochemicals Corporation were used throughout. The preparation and purification of poly-α-amino acids[29] not described in the Experimental part are given in Table I. In all cases the conditions of purification would remove any hydantoin-3-acetic acid derivatives, if present. This was proved in the cases of poly-L-phenylalanine (sample 4) and poly-DL-alanine (sample 19) by addition of authentic samples of the respective hydantoin-3-acetic acid derivatives and their quantitative removal under the conditions used for the purification of the polymers. The hydantoin derivatives were estimated colorimetrically, as described below.

N-Carboxy-DL-phenylalanine anhydride was prepared according to Farthing.[30]

N-Carboxy-L-phenylalanine anhydride (I) was prepared analogously from L-phenylalanine; yield 92%. The anhydride crystallizes from ethyl acetate in long needles, m.p. 95–96° dec., $[\alpha]^{20}D$ −25.0° (*c* 9 in dimethylformamide).

Anal. Calcd. for $C_{10}H_9NO_3$: C, 62.8; H, 4.7; N, 7.3; mol. wt., 191.2. Found: C, 62.9; H, 4.6; N, 7.2; neut. equiv., 191.[6]

Poly-L-phenylalanine was prepared by the polymerization of twice recrystallized N-carboxy-L-phenylalanine anhydride either in bulk at 120° in a high vacuum (10^{-4} mm.), or in solution (*cf.* Table I). The polymer was purified by washing with hot dimethylformamide and ether.

Anal. Calcd. for $(C_9H_9NO)_n$: C, 73.5; H, 6.2; N, 9.5. Found: C, 74.1; H, 6.6; N, 9.8.

Poly-L-phenylalanine (*n* average 32) is soluble in hot dichloroacetic acid, insoluble in hot dimethylformamide, glacial acetic acid and phenol. It is soluble in a cold 33% solution of hydrogen bromide in glacial acetic acid. Hydrolysis of poly-L-phenylalanine in concentrated hydrochloric acid in a sealed ampoule at 110–120° for 48 hours gave a quantitative yield of chromatographically and optically pure L-phenylalanine.

(22) J. W. Breitenbach and K. Allinger, *Monatsh.*, **84**, 1103 (1953).

(23) K. Heyns and R. Brockmann, *Z. Naturforschung*, **9b**, 21 (1954).

(24) L. A. Ae. Sluyterman and B. Labruyère, *Rec. trav. chim.*, **73**, 347 (1954).

(25) E. Fischer, *Ber.*, **36**, 2094 (1903). The correct structure was assigned by F. Wessely, K. Schlögl and G. Korger, *Monatsh.*, **83**, 1156 (1952).

(26) M. Frankel and E. Katchalski, THIS JOURNAL, **65**, 1670 (1943).

(27) E. Pacsu and E. J. Wilson, Jr., *J. Org. Chem.*, **7**, 124 (1942).

(28) E. Katchalski, Y. Shalitin and M. Gehatia, THIS JOURNAL, **77**, 1925 (1955).

(29) The authors wish to acknowledge the technical assistance of Israel Jacobson in the preparation of the poly-α-amino acids.

(30) A. C. Farthing, *J. Chem. Soc.*, 3213 (1950).

N-Carboxy-DL-alanine anhydride was prepared according to Bailey[31]; yield 88%; twice recrystallized from ethyl acetate–petroleum ether; m.p. 60° dec. (Bailey[31] reports 45–46°).

Anal. Calcd. for $C_4H_5NO_3$: C, 41.7; H, 4.4; N, 12.2; mol. wt., 115.1. Found: C, 41.2; H, 4.7; N, 12.1; neut. equiv., 115.[6]

Carbonyl-bis-(ε,N-carbobenzoxy-L-lysine Benzyl Ester) (V).—A 20% solution of phosgene in toluene (15 ml.) and 6 *N* sodium hydroxide (12 ml.) were added at −5 to 0° with vigorous stirring to a mixture of ε-carbobenzoxy-L-lysine benzyl ester hydrochloride[32] (15 g.) in 2 *N* sodium hydroxide (15 ml.) and toluene (20 ml.). The flow of sodium hydroxide was regulated so that the mixture remained alkaline. Ethyl acetate (200 ml.) was added to dissolve the precipitate formed, the organic layer was separated, washed with water, dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crystalline residue was washed with petroleum ether and recrystallized from toluene and from benzene; yield 86%, m.p. 138°, $[\alpha]^{20}D$ −12.0° (*c* 8 in glacial acetic acid).

Anal. Calcd. for $C_{43}H_{50}N_4O_9$: C, 67.3; H, 6.6; N, 7.3. Found: C, 67.6; H, 6.5; N, 7.5.

α,N,α′,N′-Carbonyl-bis-(L-lysine Benzyl Ester Hydrobromide) (VII).—Carbonyl-bis-(ε,N-carbobenzoxy-L-lysine benzyl ester) (V) (5 g.) was dissolved in a 33% solution of anhydrous hydrogen bromide in glacial acetic acid[16] (25 ml.). The evolution of carbon dioxide had ceased within 15 minutes at room temperature. Anhydrous ether (200 ml.) was added, the semi-solid precipitate formed was redissolved in the minimum amount of methanol, and reprecipitated with ether (200 ml.). The precipitate solidified on repeated trituration with anhydrous ether, and was dried *in vacuo* over sulfuric acid and potassium hydroxide; yield 72%. VII is very hygroscopic.

Anal. Calcd. for $C_{27}H_{40}N_4O_5Br_2$: N, 8.5; Br, 24.2. Found: N, 8.3; Br, 24.8.

The chloroplatinate of VII was prepared in aqueous solution; m.p. 205–207°. It is sparingly soluble in water.

Anal. Calcd. for $C_{27}H_{40}N_4O_5PtCl_6$: N, 6.2; Pt, 21.6. Found: N, 6.7; Pt, 22.1.

α,N,α′,N′-Carbonyl-bis-L-lysine (VIII).—The hydrobromide VII was hydrogenated at atmospheric pressure in aqueous solution in the presence of a platinum catalyst (5% platinum-on-charcoal). After the theoretical amount of hydrogen had been taken up, the catalyst was filtered off, the solution neutralized with ammonia and evaporated to dryness. The residue was triturated with acetone, and solidified on the addition of methanol. The carbonyl-bis-L-lysine was purified by precipitation from its aqueous solution by methanol; m.p. 235–240°.

Anal. Calcd. for $C_{13}H_{26}N_4O_5 \cdot 2H_2O$: C, 44.1; H, 8.5; N, 15.8; amino-N, 7.9; Br, 0; equiv. wt., 177.2. Found: C, 43.7; H, 9.0; N, 16.1; amino-N, 8.1 (Van Slyke); Br, 0.0; neut. equiv., 175, determined by anhydrous titration with perchloric acid in glacial acetic acid, using crystal violet as indicator; neut. equiv., 175, determined by anhydrous titration with sodium methoxide in benzene–methanol, using thymol blue as indicator.[33]

Carbonyl-bis-L-lysine (VIII) is soluble in water, sparingly soluble in methanol, and insoluble in acetone, ether and petroleum ether. VIII gives a positive ninhydrin reaction and a negative picric acid test. On a chromatogram developed with *n*-butyl alcohol–acetic acid–pyridine–water (15:3:10:12)[13] it shows R_f 0.16 or R_x 0.67 as compared with L-lysine.

The chloroplatinate of VIII crystallized out from aqueous methanol; m.p. 199–201°.

Anal. Calcd. for $C_{13}H_{28}N_4O_5PtCl_6$: Pt, 26.8. Found: Pt, 26.8.

L,L-α,5-Di-(4-aminobutyl)-hydantoin-3-acetic Acid (VI) Dihydrochloride. (a) **From V.**—Carbonyl-bis-(ε,N-carbobenzoxy-L-lysine benzyl ester) (V) (7.7 g.) was refluxed in concentrated hydrochloric acid–glacial acetic acid (1:1, v./v.) (50 ml.) for one hour. After the removal of solvent the residue was taken up in water (20 ml.) and the solution was repeatedly washed with ether to remove benzyl chloride. The aqueous solution was evaporated to dryness and the residue solidified on trituration with acetone. A white hygroscopic powder was obtained after drying *in vacuo* over phosphorus pentoxide and potassium hydroxide; yield 3.5 g. (95%), m.p. 214–216°, $[\alpha]^{20}D$ −50.7° (*c* 3 in water).

Anal. Calcd. for $C_{13}H_{26}N_4O_4Cl_2$: C, 41.8; H, 7.0; N, 15.0; amino-N, 7.5; Cl, 18.2. Found: C, 41.2; H, 7.2; N, 14.7; amino-N, 7.2 (Van Slyke); Cl, 18.3.

VI gives a positive ninhydrin reaction and a positive picric acid test. On a chromatogram developed with *n*-butyl alcohol–acetic acid–pyridine–water (15:3:10:12)[13] it shows R_f 0.24 or R_x 1.0 as compared with L-lysine.

(b) **From Carbonyl-bis-L-lysine (VIII).**—A solution of VIII in concentrated hydrochloric acid was brought to dryness on a water-bath, and the residue dried *in vacuo* over phosphorus pentoxide and potassium hydroxide. The product obtained was identical with the one described above (procedure a), and both gave the same chloroplatinate (m.p. 235°; found Pt, 27.1; calcd. Pt, 27.5).

Hydrolysis of VI in boiling 2 *N* sodium hydroxide for one minute yielded, after acidification with acetic acid and addition of methanol and acetone, crystals of carbonyl-bis-L-lysine (VIII).

Carbonyl-bis-(DL-alanine *n*-butylamide) was prepared by heating carbonyl-bis-(DL-alanine ethyl ester)[11] in *n*-butylamine for one minute. The amide crystallized on cooling in long needles, m.p. 225°; picric acid test positive.

Anal. Calcd. for $C_{15}H_{30}N_4O_3$: N, 17.8. Found: N, 17.6.

The salt of N-carboxytriglycine methyl ester with triglycine methyl ester was prepared from triglycine methyl ester[27] in dry chloroform by passing a stream of dry carbon dioxide through the solution (*cf.* preparation of the salt of N-carboxyglycine methyl ester with glycine methyl ester[26]). The crystalline salt began to precipitate immediately. The stream of gas was continued until the solvent had evaporated.

Anal. Calcd. for $C_{15}H_{26}N_6O_{10}$: N, 18.7; CO_2, 9.8. Found: N, 18.5; CO_2, 9.5, determined by measuring the amount of carbon dioxide liberated on addition of dilute sulfuric acid in a manometric Van Slyke apparatus.

The above substance, heated for 4 hours at 110°, gave a negative picric acid test.

Reaction of N-Carboxy-L-phenylalanine Anhydride with L-Phenylalanine Methyl Ester.—A solution of N-carboxy-L-phenylalanine anhydride (I) (0.5 g.) in anhydrous dioxane (10 ml.) was added slowly to a solution of L-phenylalanine methyl ester (1.6 g.) in anhydrous dioxane (20 ml.). After 24 hours 6 *N* hydrochloric acid (5 ml.) was added, and the solution brought to dryness on a water-bath. Ethyl acetate (20 ml.) and dilute hydrochloric acid (20 ml.) were added to the residue, and the layers were separated. The aqueous and organic layers were washed with ethyl acetate and water, respectively. The combined ethyl acetate solutions yielded, after evaporation, 18 mg. of L,L-α,5-dibenzylhydantoin-3-acetic acid[7] (IV), m.p. 210° not depressed on admixture of an authentic sample (m.p. 212°).

Isolation of L,L-α,5-Dibenzylhydantoin-3-acetic Acid from the Hydrolysate of Poly-L-phenylalanine.—Poly-L-phenylalanine (1.9 g., sample 4 of Table I) was dissolved in dichloroacetic acid (10 ml.) and concentrated hydrochloric acid (10 ml.) was added, whereupon precipitation occurred. The mixture was heated under reflux at 115° during 2 hours, and the solid was filtered off. The solution was brought to dryness, ethyl acetate (20 ml.) and dilute hydrochloric acid were added to the residue, and the layers were separated.

The organic layer was washed twice with a saturated sodium chloride solution, dried over sodium sulfate and evaporated. The semi-solid residue was triturated with anhydrous ether, dissolved in ethanol and the solution diluted with water until turbid. The crystalline L,L-α,5-dibenzylhydantoin-3-acetic acid (IV) (78 mg.) was filtered off and dried. It had a m.p. of 206° which was not depressed on admixture of an authentic sample (m.p. 212°). The product gave a positive picric acid test and a negative ninhydrin reaction.

The aqueous layer gave a negative picric acid test and a postive ninhydrin reaction.

In a separate experiment it was demonstrated that L-phenylalanine anhydride is not extracted into ethyl acetate

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

(32) B. F. Erlanger and E. Brand, THIS JOURNAL, **73**, 4025 (1951).

(33) J. S. Fritz and N. M. Lisicki, *Anal. Chem.*, **23**, 589 (1951).

under the above conditions, and cannot therefore be responsible for the appearance of a positive picric acid test.

Isolation of the α,5-Dibenzylhydantoin-3-acetic Acids from the Hydrolysate of Poly-DL-phenylalanine.—Poly-DL-phenylalanine (1.5 g., sample 1 of Table I) was hydrolyzed in a mixture of concentrated hydrochloric acid–glacial acetic acid (1:1) for 3 hours at 110°. The hydrolysate was treated as above, and the residue obtained after the evaporation of the organic layer was recrystallized from ethanol; m.p. 225° (yield 17 mg.). The mother liquor yielded on concentration 13 mg. of crystals, m.p. 165° (after sintering at 90–100° and resolidification). Authentic samples of the two racemates of α,5-dibenzylhydantoin-3-acetic acid[9] melted at 225° and 170° (sintering at 90–100° and resolidification), and caused no depression in m.p. when mixed with the corresponding substances obtained from the hydrolysate.

Isolation of the α,5-Dimethylhydantoin-3-acetic Acids from the Hydrolysate of Poly-DL-alanine.—Poly-DL-alanine (0.72 g., sample 18 of Table I) was hydrolyzed and worked up as above. The semi-solid residue obtained after evaporation of the organic layer crystallized on trituration with petroleum ether; yield 30 mg. of a mixture of both racemates of α,5-dimethylhydantoin-3-acetic acid, m.p. 135–140° (identical with that of a 1:1 mixture of authentic samples of the racemates[10]).

Titration of Poly-α-amino Acids in Anhydrous Solvents.—Dimethylformamide was shaken with anhydrous potassium carbonate and distilled at atmospheric pressure through a 80-cm. packed column (b.p. 153–154°). Blank titrations of 1-ml. samples should consume less than 0.01 ml. of 0.1 *N* sodium methoxide in methanol–benzene, and less than 0.01 ml. of 0.1 *N* perchloric acid in dioxane. Thymol blue (0.05 g.) was dissolved in 100 ml. of dimethylformamide; crystal violet (0.01 g.) was dissolved in 100 ml. of glacial acetic acid; 0.1 *N* sodium methoxide solution was prepared according to Fritz and Lisicki,[33] but using equal volumes of methanol and benzene; 0.1 *N* perchloric acid solutions were prepared by adding 16.4 g. of 70% perchloric acid to 1 l. of glacial acetic acid or to 1 l. of anhydrous dioxane.

(a) **Titration of Acid Groups.**—A sample of 5 to 20 mg. of the polyamino acid was weighed into a small test-tube (75 mm. length, 8 mm. inner diameter), dissolved (or suspended) in 0.5 ml. of dimethylformamide, and one drop of thymol blue solution was added. The solution was titrated to a deep blue end-point with 0.1 *N* sodium methoxide. The titrant was delivered from an Agla Micrometer Syringe (Burroughs Wellcome & Co., London), the tip of which dipped into the solution. The close fit between the syringe and the test-tube gives sufficient protection from atmospheric carbon dioxide. Stirring was effected magnetically, with a piece of iron wire, 5 mm. in length, made from a paper clip, serving as stirrer.

(b) **Titration of Basic Groups.**—The titrations were carried out as above. Solutions in glacial acetic acid were titrated to a blue end-point with 0.1 *N* perchloric acid in glacial acetic acid, using crystal violet as indicator. Solutions in dimethylformamide were titrated to a pink end-point with 0.1 *N* perchloric acid in dioxane, using thymol blue as indicator. End-points were somewhat sharper, and blanks were smaller, with the former method.

Colorimetric Determination of Hydantoin-3-acetic Acid Derivatives.—One ml. of a saturated aqueous solution of picric acid and 2 ml. of 1 *N* sodium hydroxide were added in a test-tube to 1 ml. of a solution containing 0.005 to 0.030 millimole of the compound. The mixture was brought to boil over a free flame and kept at 100° for 10 minutes. After cooling under the tap, 1 ml. of glacial acetic acid was added, the solution was diluted to 25 ml., and filtered, if necessary. The absorption was determined within a few hours at 5000 Å. in a 1-cm. cell against water. The molar concentration, *c*, of the final solution was calculated from $c = d/930$, where *d* is the optical density read.

In order to determine the ureido groups in purified poly-α-amino acids, the polymers were hydrolyzed for 4 to 6 hours in concentrated hydrochloric acid–glacial acetic acid (1:1) at 110°, and the partial hydrolysate was concentrated *in vacuo*. The content of hydantoin-3-acetic acid derivatives was determined as above.

All the α-amino acids whose polymers have been investigated, as well as their N-carboxyanhydrides, give a negative picric acid reaction. Various samples of poly-α-amino acids gave picric acid reactions from negative to slightly positive. Lysine peptides[14] (lysyllysine, trilysine, tetralysine, pentalysine) give a negative picric acid reaction. Carbonyl-bis-lysine (VIII), carbonyl-bis-alanine[11] and carbonyl-bis-phenylalanine[7] also give a negative reaction. Carbonyl-bis-(lysine benzyl ester) (VII) and carbonyl-bis-(alanine ethyl ester),[11] as well as carbonyl-bis-DL-alanine dibutylamide and carbonyl-(glycine)-(glycylglycine)[25] give a positive picric acid reaction.

Spectrophotometric Determination of the Tyrosine Residue.—In poly-α-amino acids prepared using tyrosine ethyl ester as the initiator of polymerization, the tyrosine content was measured in 1-cm. quartz cells on aqueous solutions at 2935 Å. and *p*H 13. In the case of polymers insoluble at this *p*H, the measurements were carried out after total hydrolysis (10 *N* hydrochloric acid, 24 hours, 120°). The molar concentration of the tyrosine residue, *c*, was calculated from $c = d/2330$, where *d* is the optical density read and 2330 is the molar extinction coefficient of tyrosine at 2935 Å. and *p*H 13.

Ultraviolet and Visual Light Absorption.—Measurements were made on a Hilger Uvispek photoelectric spectrophotometer H 700/303, at approximately 25°.

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