Studies on Dehydroalanine Derivatives. II. Synthesis of Polydehydroalanine

By Shumpei SAKAKIBARA

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In the preceding paper of this series[1] the synthesis and properties of *N*-carboxy dehydroalanine anhydride (I) were described. The present investigation was designed to synthesize polydehydroalanine using the anhydride I as starting material, and to obtain some information concerning the structure of the polypeptide.

Synthesis of Polydehydroalanine. — It was previously observed, that the carbamic acid II, which formed from the *N*-carboxy anhydride I, after hydrolysis or aminolysis was unusually stable as compared with those derived from ordinary amino acids[1]. Therefore, it was expected that the rate of polymerization of the *N*-carboxy anhydride I would be much slower than that of usual *N*-carboxy anhydrides at ordinary temperature. In order to ascertain this expectation, the rate of polymerization of *N*-carboxy dehydroalanine anhydride (I) was compared with that of *N*-carboxy DL-alanine anhydride at 30°C using cyclohexylamine as initiator. As can be seen from Fig. 1, the time-conversion curve of the polymerization of the *N*-carboxy anhydride I reached the 25% line only after 5 hours, and then very slowly approached the 35% line, while the *N*-carboxy DL-alanine anhydride polymerized very quickly, and the curve arrived at the 90% line within half an hour. It was concluded from these findings that the polymerization reaction of the *N*-carboxy anhydride I should be carried out at sufficiently high temperatures to permit a more rapid rate of the decarboxylation reaction, and that tertiary amines should be used as initiators. The tertiary amine used, would overcome the inclination for low conversion of the polymerization reaction which may be induced by the formation of ureide III as the termination reaction. A rapid rate of the polymerization reaction also seemed to be desirable in order to suppress a vinyl-type polymerization.

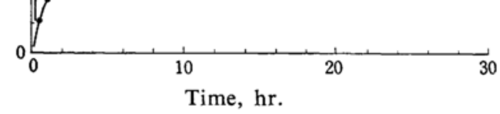
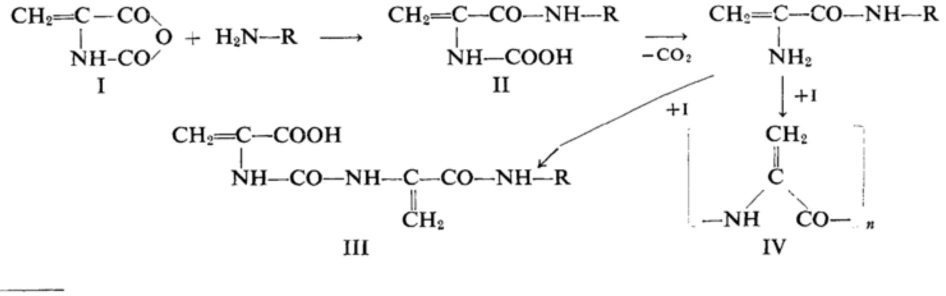


Fig. 1. Polymerization rates of *N*-carboxy anhydrides at 30±0.1°C in dioxane containing cyclohexylamine (10 mol. % against the *N*-carboxy anhydrides): I. *N*-Carboxy dehydroalanine anhydride; II. *N*-Carboxy DL-alanine anhydride.

In the present experiment, polydehydroalanine was prepared by heating a dry pyridine solution of the substance I, at 100°C, or by adding a small amount of triethylamine into a boiling dry toluene solution of the substance I. In the case of the former, the reacting solution gradually became dark-brown as the polymerization reaction proceeded and brown precipitates (IVa) formed. Evolution of carbon dioxide almost ceased after about 2 hr. In the case of the latter, the starting, slight-yellow, clear solutidn suddenly turned into brown, when a small amount of triethylamine was added into,



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[1] S. Sakakibara, This Bulletin, 32, 13 (1959).

the solution. At the same time a vigorous evolution of carbon dioxide was observed and brown precipitates gradually formed. This precipitates were partially insoluble in water, and the water soluble part was collected as polydehydroalanine (IVb).

Properties of the Polydehydroalanine. — The freshly formed brown product IV (IVa or IVb) was completely soluble in water, formic acid and dichloroacetic acid, slowly dissolved in dimethylformamide and *m*-cresol, but after complete drying it became partially insoluble in these solvents. Furthermore, about 80% of the substance IV was converted into a water-insoluble form, when preserved under ordinary conditions for about six months. Such a solubility behavior of the substance IV was similar to that of poly-DL-alanine[2,3]. It was, however, found that 34% of the aged substance IV, was completely insoluble even in formic acid, in which the aged poly-DL-alanine is readily soluble[3]. This fact seems to indicate that the substance undergoes not only α-β-configuration change of the polypeptide-bonds, but also the intermolecular cross-linkage formation due to its double bonds. After reprecipitation from water-ethanol, the polypeptide IV was still brown in color, and its ultraviolet absorption spectrum (Fig. 2) suggested the presence of some kinds of conjugated double bonds. When the polypeptide was hydrogenated as described in a later part of this paper, this absorption bands at $230\sim350$ mμ corresponding to the conjugated double bonds vanished and the brown color extremely reduced at the same time.

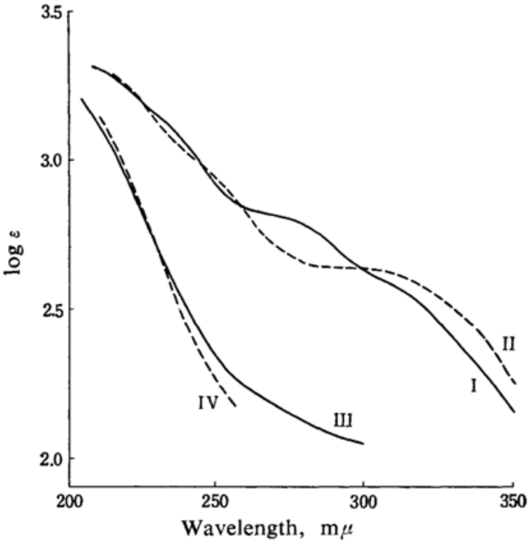

Fig. 2. Ultraviolet absorption spectra recorded on a Beckman DK-2 spectrophotometer: I. Polydehydroalanine (PDA) dissolved in distilled water; II. PDA dissolved in aq. 1 N NaOH solution; III. Hydrogenated PDA dissolved in water; IV. Poly-DL-alanine dissolved in water.

The determination of the molecular weight of the polymer was very difficult. The end-amino group of the polypeptide should not be expected to be present in this material, because of the instability of the *N*-terminal dehydroalanyl group[1]. Therefore, amino-nitrogen determination for calculating the mean molecular weight of the polymer is not significant. When this polymer was titrated with sodium methoxide for determining the end-carboxylic acid, equivalent weights of 330 and 435 were obtained, which corresponded to about 4.7 and 6.2 monomer residues, and these values were much smaller than those calculated hereafter from the expected molecular weight of the polymer. This fact suggested the following two possibilities: (1) Enolization of peptide bonds, which has been mentioned previously by Bergmann, et al. in the cases of dehydroalanyl peptides or diketopiperazines[4]. (2) Partial hydrolysis of pyrolidone rings (VI) which may be formed spontaneously from the dehydroalanyl residues as described in a later part of this paper. The delicate difference between the ultraviolet absorption spectrum of the polymer in 1 N sodium hydroxide solution and that in distilled water would also suggest the fact of enolization. Viscosities of 0.5% dichloroacetic acid solutions of these polymers, IVa and IVb, were determined at 25±0.01°C, and $\eta_{sp}/C=1.8$ and 0.9 (C expressed in base mol./l.) were obtained, respectively. If an assumption is permitted, that this polymer behaves quite similarly to poly-DL-alanine in dichloroacetic acid, the molecular weight of the polymer may be determined by comparison of the viscosity number with that of known poly-DL-alanine under the same conditions[5]. From the above consideration, about $7000\sim5000$ and $4000\sim2000$ were calculated for their molecular weights, respectively.

Hydrogenation and Hydrolysis of Polydehydroalanine for Elucidation of the Actual Structure. —Elucidation of the structure of the polymer was very difficult because double bonds involved in the molecule are expected to be reactive and they may interact with one another within the molecule. If the structure of the polymer is exactly identical with the structural formula

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[2] C. H. Bamford, A. Elliott and W. E. Hanby, "Synthetic Polypetide", Academic Press, New York (1956), p. 312.

[3] A. Elliott, *Nature*, **170**, 1066 (1952).

[4] M. Bergmann and A. Miekeley, *Ann.*, **458**, 40 (1927).

[5] C. H. Bamford, A. Elliott and W. E. Hanby, "Synthetic Polypeptide", Academic Press, New York (1956), pp. 299, 314.

IV, poly-DL-alanine should be derived as a result of hydrogenation of the polymer. An attempt of catalytic hydrogenation of the polymer IV under the presence of palladium-charcoal was unsuccessful, since the polymer was adsorbed on the catalyst and separation of the catalyst from the product was very difficult. Recently, Izumi[6] has published an excellent catalyst, which was prepared by reduction of palladium chloride co-ordinated with silk-fibroin. When this catalyst was used, the hydrogenation reaction of the polymer IV, proceeded successfully, and the starting brown solution of the polymer IV turned into a slight-yellow one, after the reaction was finished. Since the palladium atoms have previously adsorbed between peptide layers of silk-protein, no more polypeptide was adsorbed on the catalyst during the reaction. From the hydrochloric acid hydrolyzate of the product V, alanine was detected in a yield of about 5% of the theoretical by the Dowex 50 column chromatography under the condtions of Moore and Stein[7] (Fig. 3).

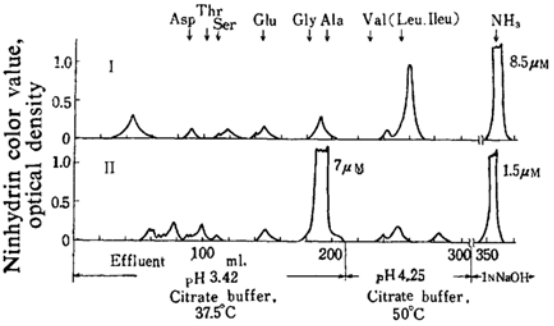
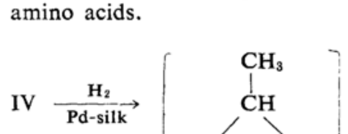


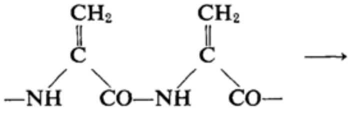
Fig. 3. Chromatographic analysis of ninhydrin-positive materials of the HCl-hydrolyzates: I. Polydehydroalanine (10 mg.); II. Catalytic hydrogenated polydehydroalanine (10 mg.). Arrows indicate the position of the peaks of standard amino acids.



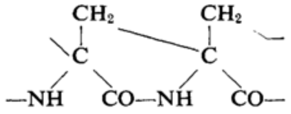
$$\mathrm{IV} \xrightarrow[\text{Pd-silk}]{H_2} \left[-NH-\underset{\displaystyle |}{\overset{CH_3}{\overset{|}{CH}}}-CO-\right]_n$$

V

The ultraviolet absorption spectrum of the hydrogenated product V (Fig. 2) was similar to that of the poly-DL-alanine and the absorption bands corresponding to the conjugated double bonds at 250∼350 mμ disappeared after hydrogenation as described previously. The infrared spectrum of the hydrogenated product V (Fig. 4), however, was neither quite different from that of the starting polymer, nor similar to that of poly-DL-alanine. This infrared spectrum resembled rather that of poly-α-amino acrylic acid[8]. Since the actual structure of the poly-α-amino acrylic acid is considered to be polypyrolidone[9], a greater part of the polymer would be composed of such a polypyrolidone-type structure VI, which might form spontaneously from the polydehydroalanine by interaction of the neighboring double bonds. The infrared absorption bands at 1700∼1760 cm^{-1} (Fig. 4) supported also the presence of five membered cyclic amide. If the structure of the polydehydroalanine corresponds to the formula IV exactly, its hydrochloric acid hydrolyzate must all be pyruvic acid and ammonia. However,



IV



VI

many kinds of ninhydrin-positive materials, the total yield of which was about 10% of the ideal formula IV, were detected by ion-exchanger column chromatography from the hydrochloric acid hydrolyzate (Fig. 3). Ammonia, which formed in this case was also directly ascertained[10] and amounted to 9.5% of the theoretical. These facts all supported the formation of the non-hydrolyzable material which was considered to be such a polypyrolidone. Actually, the hydrochloric acid hydrolyzate of the polymer IV became dark brown, and this fact indicated a formation of huminic material during the course of the reaction. From the result of the experiments and the above considerations, it may be appropriate to consider that the obtained polymer IV, consists of about 90% of polypyrolidone-type polypeptide and about 10% of dehydroalanyl residues. In other words, the synthesis of polydehydroalanine, which was exactly expressed as the structural formula IV, was unsuccessful in the present experiment. If the preparation of co-polymer of dehydroalanine and another stable amino acid is possible, the interaction of the neighboring double bonds would decrease more

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[6] Y. Izumi, This Bulletin, 32, 932 926, 942 (1959).

[7] S. Moore and W. H. Stein, *J. Biol. Chem.*, **192**, 663 (1951).

[8] This material was prepared from a vinyl-type polymer of the substance I or from that of α-phthalimido acrylc acid as will be described in a later communication of the series.

[9] cf. M. Vrancken and G. Smets, *J. Polymer Sci.*, **14**, 521 (1954).

[10] J. E. Varner, et al., *Anal. Chem.*, **25**, 1528 (1953).

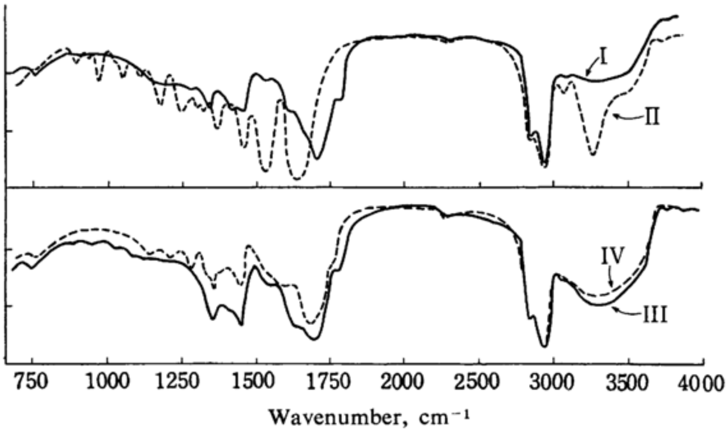

Fig. 4. Infrared absorption spectra recorded on an Adam-Hilger H-80 infrared spectrophotometer: I. Polydehydroalanine; II. Poly-DL-alanine; III. Hydrogenated polydehydroalanine; IV. Poly-α-amino acrylic acid. Nujol suspension.

distinctly. A communication on the reactivity of the dehydroalanyl groups involved in the present polymer will be published in the near future.

Experimental

Materials.—*N*-Carboxy dehydroalanine anhydride (I) and *N*-carboxy DL-alanine anhydride were prepared according to the description in the previous paper[1].

Determination of the Polymerization Rate of I.—The substance I (350 mg.) was dissolved in freshly distilled dry dioxane (15.00 ml.) containing cyclohexylamine (30.4 mg.). The solution was divided into 14 test tubes so that each tube contains 1.00 ml. of the solution, and the tubes were sealed. Then, they were allowed to react in a bath at 30± 0.1°C, simultaneously. Each of the portions was opened successively at times and dry nitrogen was introduced for about one min. into the solution, which was then titrated with a methanol-benzene solution of sodium methoxide, using thymol blue as an indicator according to the description of Berger, et al.[11] (Fig. 1). The sodium methoxide solution (ca. 0.3 N) was standardized every 5 hr. with purified benzoic acid. *N*-Carboxy DL-alanine anhydride was subjected to the reaction under the same condition described above (Fig. 1).

Polymerization of I in Pyridine (Preparation of Polydehydroalanine IVa).—A solution of I (5 g.) in purified dry pyridine (15 ml.) was heated in a boiling water bath for about 2 hr. After cooling to room temperature, the reddish brown reaction mixture was poured into ethyl acetate (100 ml.). The precipitated brown material was centrifuged, washed thoroughly with ethyl acetate and ether, and dried. The brown powder obtained (3.05 g.) was reprecipitated from water-ethanol and dried under reduced pressure at room temperature. The yield was 2.4 g. (64%).

Found: C, 43.8; H, 5.76; N, 15.5; volatile part at 135°C, 17.9. Calcd. for $(C_3H_3ON+14/18\cdot H_2O+1/20\cdot C_2H_6O \text{ (ethanol)})_n$: C, 43.7; H, 5.71; N, 16.5; volatile part, 18.8%.

Found*: C, 49.50; H, 4.24; N, 18.86; ash, 1.2%; Na-equiv. wt.[12], 330. Calcd. for $(C_3H_3ON)_n$: C, 52.17; H, 4.35; N, 20.3%.

This material was hard to burn up for analysis.

Polymerization of I in Toluene (IVb).—Few drops of triethylamine were added into a boiling toluene solution of I (1 g./20 ml.). The starting clear solution, which was light-yellow, suddenly turned into brown as the reaction occurred and reddish brown precipitates formed immediately under the vigorous evolution of carbon dioxide. After about 30 min., the reaction mixture was cooled and the formed precipitates were filtered off, washed three times with ethyl acetate and dried. The yield was 0.62 g. This material was extracted with sufficient amount of water. The water extract was concentrated to about 5 ml. and brown powder (0.19 g.) (IVb) was reprecipitated with ethanol. The water insoluble part of the material was about 0.18 g. Na-equiv. wt. of the material IVb was 435.

Hydrogenation of IVa.—The substance IVa (300 mg.) was dissolved in water (50 ml.) and hydrogenated in an autoclave under 100 atm. of hydrogen at 50～60°C for about 8 hr. using palladium-silk catalyst (200 mg.). After the reaction was complete, the catalyst was filtered off and the filtrate was concentrated to about 10 ml. Ethanol (100 ml.) was added into the concentrate and about 160 mg. of light-yellow powder was precipitated. This material was reprecipitated from water-ethanol and dried under reduced pressure at 118°C.

Found: C, 47.67; H, 5.13; N, 16.68. Calcd. for $(9/10\cdot C_3H_3ON+1/10\cdot C_3H_5ON+2/5\cdot H_2O)_n$: C, 47.1; H, 5.36; N, 18.3%.

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

[12] M. Sela and A. Berger, *J. Am. Chem. Soc.*, 77, 1893 (1955).

*) This material was dried over phosphorus pentoxide under reduced pressure at 135°C, in which procedure a certain extent of decomposition might occur, such as liberation of formaldehyde or ammonia.

The hydrogenated material (10 mg.) was hydrolyzed with 6 N hydrochloric acid for about 20 hr. and the hydrolyzate was concentrated to dryness. The residue was then subjected to column chromatography under the Stein-Moore's condition (Fig. 3). The fraction corresponding to alanine was collected, treated with 2,4-dinitrofluorobenzene and purified by celite column chromatography using ether-phosphate buffer (0.25 M, pH 6.5) as a developer. Infrared spectrum of the purified main fraction was agreed with that of authentic DNP-alanine.

Summary

The polymerization rate of *N*-carboxy dehydroalanine anhydride (I) was compared with that of *N*-carboxy DL-alanine anhydride, and it was found, that the former was much slower than the latter at 30°C. Polydehydroalanine (IV) was obtained by heating the substance I in dry pyridine at 100°C, or by adding a small amount of triethylamine into the boiling toluene solution of I. The substance IV was water-soluble brown powder, the actual structure of which was supposed to be a pyrolidone-type polypeptide VI containing about 10% of dehydroalanyl residues.

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Institute for Protein Research
Osaka University
Nishi-ku, Osaka