SYNTHESIS OF POLY-L-ARGININE AND THE STATISTICAL COPOLYMER OF L-ARGININE, L-LYSINE, AND NEUTRAL AMINO ACIDS

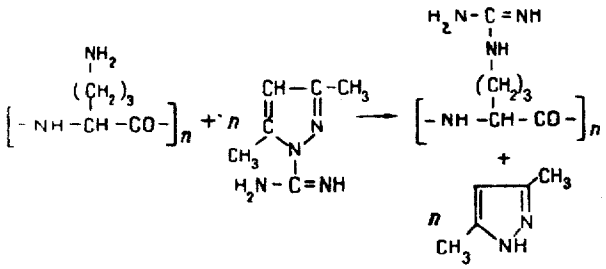
V. G. Debabov, V. D. Davydov, and Yu. N. Kosaganov

Khimiya Prirodnykh Soedinenii, Vol. 2, No. 3, pp. 195–200, 1966

Synthetic polypeptides play an important role in the investigation of the secondary structure of proteins [1]. Moreover, polypeptides containing basic amino acids are of independent interest as models of protamines and histones of natural proteins of strongly basic nature.

In the present paper we consider the synthesis of poly-L-arginine and the statistical copolymer comprising L-arginine, L-lysine, L-alanine and glycine. The latter synthesis is important from the point of view of the possibility of obtaining polypeptides containing lysine and arginine simultaneously.

Up to the present time, only poly-DL-arginine obtained by Katchalski [2] in 1951 by the guanidation of poly-DL-ornithine with S-methylisothiourea, has been described in the literature. The guanidation was carried out in methanolic solution in the presence of sodium methoxide. Our attempts to repeat this method both with L- and with DL-polyornithine, led to products with a low degree of guanidation. As guanidating agent we used 3,5-dimethyl-1-guanylpyrazole [3]. The possibility of using this reagent for the guanidation of proteins has been demonstrated previously [4]. The guanidation reaction takes place in aqueous solution at pH < 9 in accordance with the following equation:



For guanidation we used samples of poly-L-ornithine that we had synthesized. Some characteristics of the initial samples are given in the table. When the reaction was carried out in water (pH 9.5) at room temperature (20 hr), we found that the degree of guanidation was 60%* and that a change in the ionic strength had a considerable influence on the course of the guanidation reaction. It was found that an increase in the ionic strength (the addition of KI to the reaction mixture up to a final concentration of 6 M) accelerated the reaction and the percentage guanidation rose (Fig. 1). As

Some Properties of Aqueous Solutions of Poly Amino Acids

Experiment no.	Poly-L-ornithine		Poly-L-arginine		
	$[\eta]_{rel}$ at c 1%	$[\alpha]_D^{20}$ at c 0.2–0.3%	$[\eta]_{rel}$ at c 1%	$[\alpha]_D^{20}$ at c 0.25%	Mol. wt.
1	0.19	−32.5	0.12	−30	3 000
2	1.02	−78	1.66	−85.5	23 000
3	1.98	−81	2.22	−89	31 000
4	3.64	−92	4.90	−98	88 000

can be seen from the figure, with potassium iodide guanidation takes place almost quantitatively (to the extent of 96–97%) and with a higher velocity. It is probable that the effect that we have observed is purely macromolecular, since in the guanidation of the monomer (L-ornithine) under the same conditions an increase in the ionic strength of solutions does not accelerate the reaction (Fig. 2).

*Here and below, molar percentages are referred to, i.e., the number of residues subjected to guanidation per 100 residues found by analysis.

When low-molecular-weight poly-L-ornithine was guanidated (sample no. 1 in the table), the final polyarginine was isolated through the flavianate derivative; in the case of the high-molecular-weight samples (nos. 2–4) the poly-L-arginine was subjected to dialysis. The latter was carried out for 48 hr against flowing distilled water, the final reaction mixture not being acidified since the hydrochloride of 3,5-dimethylpyrazole is sparingly soluble in water. The molecular weights of the various samples of poly-L-arginine were determined in a "Spinco E" ultracentrifuge by the method proposed by Ehrenberg [5] using schlieren optics from the distribution of the concentration of the substance in the meniscus in accordance with the formula:

$$M = \frac{\left(\frac{\partial e}{\partial x}\right)_m}{\omega^2 x_m c_m (1 - \bar{V}\rho)},$$

where x_m is the distance from the meniscus to the axis of rotation; ρ is the density of the solvent, taken as unity; $\bar{V}$ is the specific partial volume of the polymer, equal to 0.76; c_m is the concentration in the meniscus; and ω is the angular velocity of rotation of the rotor.

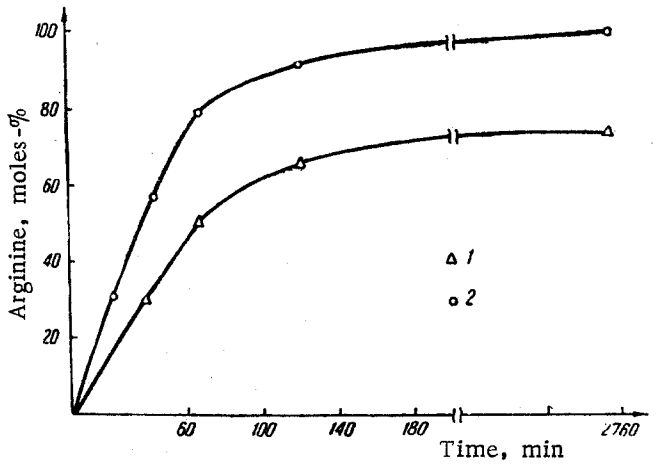


Fig. 1. Guanidation of poly-L-ornithine in 1) aqueous solution and 2) 6 M potassium iodide.

The characteristic curve of the distribution dc/dx in the cell, from which $\left(\frac{de}{dx}\right)_m$ is determined for the given conditions, is shown in Fig. 3. The initial concentration of the polymer in 1% NaCl solution was 5 mg/ml in all experiments. The molecular weights of the samples of poly-L-arginine are shown in the table.

One of the objects of the present work was to develop the synthesis of a copolymer containing L-lysine and L-arginine simultaneously with neutral amino acids.

We obtained the polymer by the combined polycondensation of the N^{α}-carboxyanhydrides of N^{δ}-carbobenzoxy-L-ornithine, N^{ε}-tosyl-L-lysine, L-alanine, and glycine in dioxane. The carbobenzoxy group was removed by means of hydrogen bromide in chloroform. In this reaction, the tosyl groups are completely stable. The polymer with the liberated amino groups of the ornithine was subjected to guanidation in 50% methanol using 3,5-dimethyl-1-guanylpyrazole. The tosyl groups were removed by means of dry hydrogen in chloroform in the presence of phenol by a method first described by Rudinger, et al., [6]. Fairly complete elimination of the tosyl group was found; at least an examination of the UV spectra of the polypeptide showed no appreciable amount of aromatic residues. The molecular weight of the polypeptide obtained, determined roughly from the relative viscosity [7], was low and of the order of 2500–3000.

Amino acid analysis of the polymers was carried out by the standard method in an automatic amino acid analyzer, the samples being hydrolyzed with 2.5 N hydrochloric acid at 120° C for 20 hr.

Experimental

Synthesis of poly-L-arginine. A solution of 0.5 g of poly-L-ornithine hydrobromide in 8 ml of water was treated with 30 g of potassium iodide (solution 1). A second solution was prepared by suspending 7 g of 3,5-dimethyl-1-guanylpyrazole nitrate (mp 161°–162° C) in 17 ml of water and the pH was brought to 9.5 with 4 N caustic soda. Then solutions 1 and 2 were mixed, the pH was brought to 9.5 and the reaction mixture was stirred for 20 hr with a magnetic stirrer at room temperature. Depending on the mean molecular weight of the poly-L-ortnithine used for the guanidation process, the subsequent working up was carried out by two methods.

The following method was used for a sample No. 1, which possessed a low molecular weight. The precipitate of poly-L-arginine that was deposited after guanidation for 20 hr was filtered off and suspended in 20 ml of water, and the suspension was acidified to pH 3–4 with 1 N hydrochloric acid, the polymer dissolving completely. A solution of 5 g of sodium flavianate in 20 ml of water was heated to + 50° C and mixed with the solution of poly-L-arginine acidified and heated to + 50° C, and the mixture was left for 12 hr in the refrigerator for crystallization. The precipitate of poly-L-arginine flavianate that was deposited was separated off, and was washed on the filter with ice-water, alcohol, and ether. The substance was dried under vacuum over P_2O_5. Yield 1.5 g, mp 198°–210° C. The poly-L-arginine flavianate (1.5 g) was mixed with 50 ml of water, and 5 ml of concentrated sulfuric acid was added. The mixture was heated to + 40°–50° C. The poly-L-arginine flavianate rapidly dissolved, and a precipitate of flavianic acid was immediately deposited from the solution. After cooling to room temperature, the flavianic acid was filtered off and the solution was extracted 3–4 times with butanol until the yellow coloration of the aqueous layer had disappeared. The colorless solution was neutralized with saturated $Ba(OH)_2$ solution to pH 6.8–7.2. The barium sulfate was separated off by centrifuging. The solution was evaporated to 15 ml in a rotary evaporator at a temperature not greater than + 50° C and was poured into 100 ml of acetone. The precipitate was separated off, washed with a small amount of ice-water and with acetone, suspended in 20 ml of water acidified to pH 3.4 with 6 N hydrochloric acid, and freeze-dried. Yield 0.36 g, $[\alpha]_D^{20}$ −30° (c 0.25; water). The amino acid composition (in micromoles) was arginine 3.24, ornithine 0.65 (no other ninhydrin-positive substances were found); relative viscosity 0.12 (c 1; water), mol. wt. 3000.

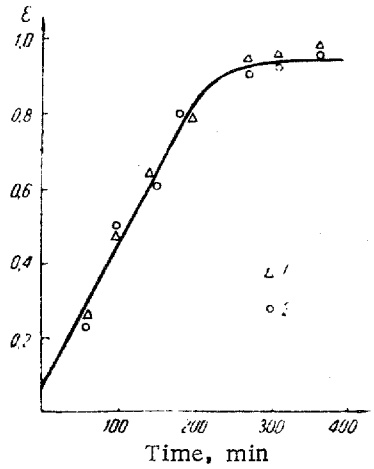


Fig. 2. Guanidation of L-ornithine in 1) aqueous solution and 2) an aqueous solution containing 6 M KI.

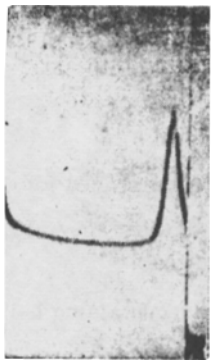


Fig. 3. Characteristic pattern of the distribution $\partial c/\partial x$ in the cell in experiments to determine the molecular weights of the poly-L-arginine (initial concentration of poly-L-arginine 5 mg/ml).

In the case of the high-molecular-weight samples, for example No. 2, the reaction mixture after guanidation was dialyzed against distilled water for 24 hr and was then acidified to pH 3–4 and dialysis was continued for 48 hr. The solution was filtered and freeze-dried. Yield 450 mg, $[\alpha]_D^{20}$ −85.5° (c 0.25; water). Amino acid composition (micromoles): arginine 3.02, ornithine 0.109; relative viscosity 1.66 (c 1; water), mol. wt. 23,000.

Synthesis of a copolymer. Polycondensation of N^{α}-carboxyanhydrides. N^{δ}-carbobenzoxy-N-$^{\alpha}$-carboxyanhydro-L-ornithine (1.1 g, 3.8 mmole) with mp 89° C (literature gives mp 86°–88° C [8]), N^{ε}-tosyl-N-$^{\alpha}$-carboxyanhydro-L-lysine (0.25 g, 0.7 mmole) with mp 97°–98° C (literature gives mp 100°–105° C [9]), N^{α}-carboxyanhydro-L-alanine (0.15 g, 1.3 mmole) with mp 92° C (literature gives mp 92° C [10]), and the N^{α}-carboxyanhydride of glycine (0.25 g, 2.4 mmole) with mp 100° C (literature gives mp 100° C [11]) were dissolved in 50 ml of absolute dioxane and, with stirring, 0.08 ml of 1.27 N sodium methoxide in methanol was added. The solution was stirred at room temperature until the evolution of CO_2 ceased (15 hr). The reaction mixture was poured into 350 ml of 96% methanol containing a few drops of concentrated hydrochloric acid. The precipitate was separated off and dissolved in 200 ml of chloroform, and the solution was filtered. The clear chloroform solution was added in drops to 1 *l* of hexane. The polymer which deposited was filtered off and dried under vacuum over P_2O_5 at + 60° C. Yield 1.2 g.

Elimination of the carbobenzoxy groups from the ornithine residues in the polymer. A current of dry hydrogen bromide was passed through a solution of 1.2 g of the polymer in 300 ml of absolute chloroform for 45 min. The solution was stirred for 2 hr. The precipitate was filtered off, washed with chloroform and absolute ether, and dried under vacuum over caustic potash. Yield 1.1 g.

Guanidation of the N^{δ}-amino groups of ornithine in the polymer. The hydrobromide of the polymer obtained (1.1 g) was dissolved in 32 ml of 50% methanol and 66 g of potassium iodide was added (solution 1). 3,5-Dimethyl-1-guanylpyrazole nitrate (14 g) was suspended in 17 ml of water, and the pH was brought to 9.5 with 4 N NaOH (solution 2). Solutions 1 and 2 were mixed, the pH was brought to 9.5, and the mixture was left at room temperature for 20 hr. The precipitate which deposited was filtered off, washed with water, mixed with 20 ml of water, and acidified to pH 3–4. The yield after freeze-drying was 1.1 g.

Elimination of the tosyl groups from the N^{ε}-amino groups of the lysine. Dry hydrogen bromide was passed for 1 hr through a suspension of 1.1 g of the polymer in 20 ml of chloroform to which 1.5 g of phenol had been added. Then the reaction mixture was heated for 4 hr in a sealed tube at + 65° C. The precipitate was filtered off and washed with chloroform and absolute ether. The polymer was reprecipitated 3–4 times from methanol with absolute ether, dried under vacuum over caustic potash, dissolved in a small amount of water, and freeze-dried. Yield 0.8 g, $[\alpha]_D^{20}$ −40.5° (c 0.35; water). Amino acid composition (micromoles): arginine 0.54, lysine 0.14, glycine 0.16, and alanine 0.08.

Study of the guanidation of poly-L-ornithine. The investigation was carried out with samples of poly-L-ornithine hydrobromide with a relative viscosity of 1.98 dialyzed against distilled water (48 hr) and freeze-dried. The guanidation was carried out as described above. In a control experiment, the concentrations of the reagents were the same but no potassium iodide was added. After predetermined intervals of time, aliquot samples were taken from the reaction medium, and these were dialyzed for 24 hr against distilled water, then acidified to 3–4, and dialyzed again for 48 hr. The samples were hydrolyzed in sealed tubes with a 30–50 fold amount of 2.5 N hydrochloric acid at 120° C for 20 hr. The ornithine and arginine contents of the hydrolyzates were determined by quantitative paper chromatography after the staining of the chromatograms with ninhydrin [12]. The system for chromatography was tert-butyl alcohol–water–formic acid (70:15:15), paper Whatman 3 mm, time of run 18 hr. The amounts of arginine and ornithine were expressed in molar percentages of the total amino acids found. No other ninhydrin-positive substances besides arginine and ornithine were found in the hydrolyzates. The results of the comparison of the reaction velocities in the presence of a salt (6 M KI) and without it can be evaluated from Fig. 1.

Investigation of the guanidation of L-ornithine. It was established in preliminary experiments that 3,5-dimethyl-1-guanylpyrazole does not give a coloration under the conditions of the Sakaguchi reaction, and therefore the velocity of the guanidation reaction can be followed by means of the increase in the coloration in the Sakaguchi reaction [13] of aliquot samples taken from the reaction mixture after predetermined intervals of time. The conditions for performing the guanidation reaction were the same as for the poly-L-ornithine. The results of a comparison of the rates of the reaction in the presence of the salt (6 M KI) and without it show (see Fig. 2) that the addition of potassium iodide to the guanidation reaction does not affect its rate.

Summary

1. A method for the quantitative guanidation of the N^{δ}-amino groups of ornithine in polypeptides has been developed. By means of this method poly-L-arginine with a high molecular weight (about 88 000) has been obtained for the first time.

2. A statistical copolymer simultaneously including L-arginine and L-lysine residues has been synthesized for the first time.

REFERENCES

1. E. Katchalski, Adv. in Protein Chem., 13, 244, 1958.
2. E. Katchalski, J. Am. Chem. Soc., 73, 3922, 1951.
3. R. Bonnard, Can. J. Chem., 36, 1541, 1958.
4. A. Habeeb, Biochem. Biophys. Acta, 34, 294, 1959.
5. A. Ehrenberg, Acta Chem. Scand., 11, 1257, 1957.
6. J. Rudinger, Coll. Czech. Chem. Comm., 4, 1174, 1955.
7. A. Garon and A. Berger, Biochem. Biophys. Acta, 69, 397, 1963.
8. R. J. Singe, Biochem. J., 42, 99, 1948.
9. R. Roeske, F. H. C. Steward, and R. J. Stedman, J. Am. Chem. Soc., 78, 5883, 1956.

10. J. L. Baily, Nature, 164, 889, 1949.
11. A. C. Farting, J. Chem. Soc., 3213, 1950.
12. K. T. Poroshin, V. G. Debabov, and V. I. Maksimov, Izv. AN SSSR, ser. khim., 6, 1136, 1961.
13. C. J. Weber, J. Biol. Chem., 86, 217, 1930.

28 June 1965

Zelinskii Institute of Organic Chemistry, AS USSR