N-Aminoalkylpolyglycine. Synthesis, Antibacterial Activity, and Electrochemical Behavior as a Titrant of Living Cells

ELEONORA SANTUCCI, MARCELLO MARCHETTI, FRANCO ALHAIQUE, CLAUDIO BOTRÉ, AND FULVIO M. RICCIERI

Istituto di Chimica Farmaceutica dell'Università di Roma, Centro di Chimica del Farmaco del C.N.R., Rome, Italy

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N-Aminoalkyl derivatives of biologically inactive homopolypeptides were synthesized as potential biologically active products. *N*-(β-Aminoethyl)polyglycine showed growth inhibitory action on *Escherichia coli*, while a random copolymer of ornithine-*N*-(β-aminoethyl)glycine was inactive. The antibacterial activity of the new polymers was studied by a turbidimetric method and by potentiometric measurements following a new technique which uses membrane electrodes. Thus, distinction between the electrostatic and nonelectrostatic contributions to the bacteria–polymer interaction was possible and permitted correlation of the electrochemical phenomenon with antimicrobial activity.

Several synthetic polyamino acids have been investigated to determine correlations between biological function and chemical structure, in view of possible chemotherapeutic implications.[1] A series of polymers has been synthesized from natural polypeptides of well-known biological activity[2] by varying the sequence and the composition of the amino acid residues of the natural products.[3] However, the forces responsible for the interactions between the polymeric molecules and the complex bacterial structures have not been defined well. The presence of specific nonelectrostatic forces of the H bond and Van der Waals bonds has been demonstrated by Katchalski, *et al.*[3c] The significance of these forces is clear, although they may not be the only ones involved.[3c]

Important determinants of the chemotherapeutic activity of natural and synthetic polyamino acids include (a) the polypeptide structure (generally, monomers do not exhibit growth-inhibiting activity) and (b) the cationic nature of basic polypeptides (neutral and acid polyamino acids generally are inactive). For example, polyglycine is unable to inhibit the growth of bacteria, whereas polyornithine has antibacterial activity.[3d]

These considerations prompted us to synthesize *N*-alkyl-substituted polymers as potentially biologically active products. In order to enhance the expected effect we increased the number of positive charges of the original polymer by introducing an NH_2 group at the end of the substituting alkyl chain. Thus the polymers described in this paper can be considered as *N*-aminoalkyl derivatives of biologically inactive homopolypeptides. The N-substitution in the polymeric chain, by introducing a structural change, should influence the nonelectrostatic forces involved in the interaction with bacteria and thus permit distinction of the electrostatic and nonelectrostatic contributions to the phenomenon.

We studied bacteria–polymer interactions by potentiometric measurements using the "coupled" membrane system described elsewhere.[4] Antibacterial activity of the new polymers was tested by following the growth of the microorganism by a turbidimetric method.[5] Thus, we were able to correlate electrochemical phenomena with antimicrobial activity.

Chemistry.—The monomer synthesis was based on the hydrolysis of the 2-ketopiperazines obtained by a ring closure reaction between ethylenediamine and an α-halo ester.[6]

Polymers were prepared through the *N*-carboxy anhydride (NCA) intermediate.[7] Some derivatives of the *N*-(β-benzyloxycarbonylaminoethyl)glycine NCA were also prepared (see Experimental Section).

The polymerization was performed either in solution or in bulk (the first method gave better results) and the protecting group was easily removed with HBr in AcOH.[8] The average degree of polymerization ($\overline{DP}$) was determined by titrating the terminal groups and by osmometric measurements. The polymers were passed through an ion-exchange resin and the concentrated solution of the free base was separated in fractions of different $\overline{DP}$ through a Sephadex column. Each fraction was then lyophilized and its molecular weight was determined in the usual way.

Experimental Section

Melting points, taken in capillary tubes in a Schmelzpunktbestimmungsapparat according to Tottoli, are uncorrected. Osmometric measurements were performed with a Knauer Dampfdruck osmometer. All analytical samples gave appropriate ir spectra and combustion analysis for C, H, N, and Cl within 0.4% of theory.

Hydrolysis of Ia and Ib.—Both 2-ketopiperazine (Ia) and 3-methyl-2-ketopiperazine (Ib)[6] (20 g, 0.2 mole of Ia, and 50 g, 0.43 mole of Ib) were treated with a large excess of concentrated HCl and gently refluxed for 3 days. The solvent was then distilled *in vacuo* and the residue recrystallized from MeOH–Et_2O. The

(1) (a) E. Katchalski, *Adcan. Protein Chem.*, **6**, 123 (1951); (b) M. Sela and E. Katchalski, *ibid.*, **14**, 391 (1959).

(2) R. L. M. Synge, *Quart. Rev., Chem. Soc.*, **3**, 245 (1949).

(3) (a) E. Katchalski, L. Bichovski-Slomnitzki, and B. E. Volcani, *Bull. Res. Counc. Israel*, **1**, 153 (1951); (b) E. Katchalski, L. Bichovski-Slomnitzki, and B. E. Volcani, *Nature (London)*, **169**, 1905 (1952); (c) E. Katchalski, L. Bichovski-Slomnitzki, and B. E. Volcani, *Biochem. J.*, **55**, 671 (1953); (d) L. Bichovski-Slomnitzki, A. Berger, J. Kurtz, and E. Katchalski, *Arch. Biochem. Biophys.*, **65**, 400 (1956); (e) M. A. Stahmann, Chemotherapeutic Possibilities of Polyamino Acids. Polyamino Acids, Polypeptides, and Proteins. M. A. Stahmann, Ed., The University of Wisconsin Press, Madison, Wis. (1962); (f) E. Katchalski, A. Berger, L. Bichovski-Slomnitzki, and J. Kurtz, *Nature (London)*, **176**, 118 (1955).

(4) (a) C. Botré, S. Borghi, and M. Marchetti, *Biochim. Biophys. Acta*, **135**, 208 (1967); (b) C. Botré, M. Marchetti, S. Borghi, and A. Memoli, *ibid.*, **183**, 249 (1969).

(5) G. Giovannozzi-Sermanni, R. Saviano, and M. Brandolini, *Ric. Sci.*, **35**, 23 (1965).

(6) S. J. Aspinall, *J. Amer. Chem. Soc.*, **62**, 1202 (1940).

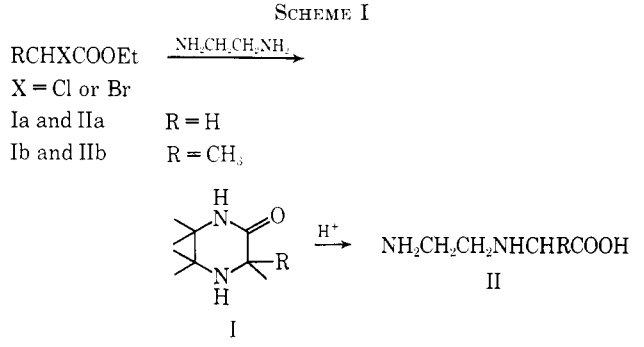
(7) E. Katchalski and P. Spitnik, *ibid.*, **73**, 2946 (1951).

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

TABLE I

No.		$\overline{DP}$	Concn (μg/ml)	Lag period: Abs time (min)	Lag period: Rel (min)	Growth period: Abs time (min)	Growth period: Rel (min)
	Test			510	1.0	170	1.0
I	Polyornithine	19	24	1880	3.6	250	1.5
II	Poly-IIa	19	60	1120	2.2	120	0.7
III	Poly-IIb	19	110	510	1.0	170	1.0
IV	Polyornithine	5	24	1740	3.4	220	1.3
V	Copolymer	5	110	510	1.0	170	1.0

SCHEME I



resulting *N*-(β-aminoethyl)glycine and *N*-(β-aminoethyl)-DL-alanine·HCl were dissolved in hot H_2O and treated with Ag_2CO_3 in a H_2O suspension. The mixture was filtered and H_2S bubbled through the solution in order to precipitate Ag^+. After filtration over activated charcoal and vacuum evaporation of the solvent, the residual yellow oil slowly solidified at room temperature. The free base (IIa) could be recrystallized from H_2O–EtOH, mp 154–155° (20.5 g, 86.7% yield). IIb was recrystallized from absolute EtOH, mp 124.5–125° (45.4 g, 80% yield). *Anal.* IIa ($C_4H_{10}N_2O_2$) C, H, N; IIb ($C_5H_{12}N_2O_2$) C, H, N.

***N*-(β-Aminoethyl)glycine Bis-CBZ Derivative.**—IIa (9 g, 0.076 mole) was dissolved in 2 *N* NaOH (IIa·2HCl may be used directly). Equivalent amounts of benzyl chlorocarbonate and 4 *N* NaOH were added simultaneously dropwise to the cooled solution and worked up according to Bergmann and coworkers.[9] The product recrystallized from Et_2O–petroleum ether had mp 77–78° (16.2 g, 55.2% yield). *Anal.* ($C_{20}H_{22}N_2O_6$) C, H, N.

***N*-(β-Aminoethyl)-DL-alanine bis-CBZ derivative** was obtained in the same way as a white gum which did not solidify (65% yield). *Anal.* ($C_{21}H_{24}N_2O_6$) C, H, N.

***N*-Carboxyanhydrides** could be prepared in good yield (82%) by treating equimolecular amounts of the bis-CBZ derivatives and PCl_5 in dry C_6H_6. The NCA derivative was precipitated by addition of petroleum ether after refrigerating overnight at 5°. *N*-(β-Benzyloxycarbonylaminoethyl)glycine-NCA, recrystallized from dry C_6H_6–petroleum ether, melted at 124–125° dec. *Anal.* ($C_{13}H_{14}N_2O_5$) C, H, N.

No crystalline product could be obtained from the corresponding DL-alanine anhydride. *Anal.* ($C_{14}H_{16}N_2O_5$) C, H, N.

***N*-(β-Benzyloxycarbonylaminoethyl)glycine and Its Me Ester.**—Anhydride (1.5 g, 5.4 mmo'es) was dissolved in 10 ml of Me_2CO and 2 ml of 5 *N* HCl. After standing overnight at room temperature the solution was concentrated and the white product obtained was dissolved in a minimum amount of H_2O. By adding dilute NH_4OH to pH 6.7 *N*-(β-benzyloxycarbonylaminoethyl)-glycine precipitated as a viscous oil which crystallized on standing at 5°. Recrystallization from EtOH gave 700 mg (52% yield) of product, mp 207–208° dec. *Anal.* ($C_{12}H_{16}N_2O_4$) C, H, N.

The corresponding Me ester·HCl was obtained by treating the same anhydride (900 mg, 3.2 mmoles) with HCl in absolute MeOH (780 mg, 80% yield; mp 134–135°). *Anal.* ($C_{13}H_{19}ClN_2O_4$ C, H, Cl, N.

Polymerization of NCA Derivatives.—*N*-(β-Benzyloxycarbonyl-aminoethyl)glycine-NCA (8 g, 0.027 mole) was refluxed for 3 days in dry PhMe or C_6H_6. The solution was allowed to cool at room temperature while the polymer (3 g) precipitated as a yellow-brown solid. The precipitate was purified by dissolving it in DMF and pouring the solution into H_2O while stirring vigorously. $\overline{DP}$ of this product, determined by titrating the terminal COOH groups with MeONa,[10] was between 18 and 20. More polymer could be obtained by vacuum distillation of the solvent.

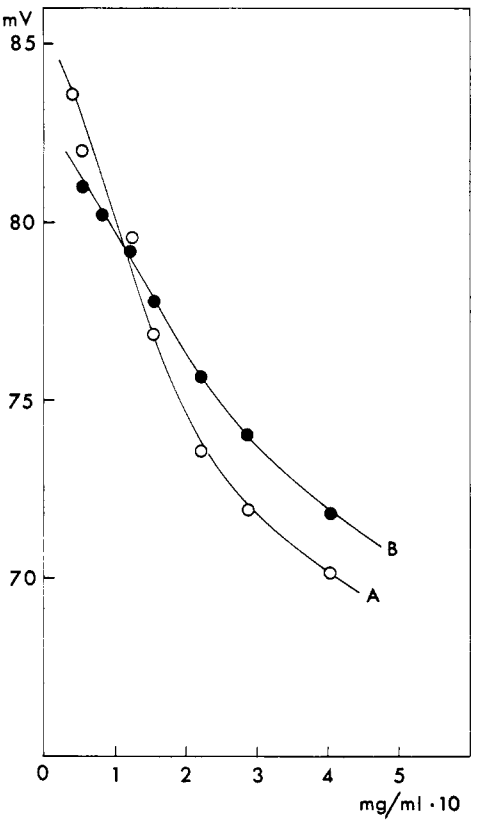


Figure 1.—Electromotive force recorded in solutions of *N*-(β-aminoethyl)polyglycine in 1 × 10^{-4} *M* KCl (DP = 19 in plot A; $\overline{DP}$ = 6 in plot B) *vs.* the polypeptide concentration.

The same procedure was followed for the polymerization of the corresponding DL-alanine derivative.

In the case of the random copolymer, ornithine-*N*-(β-aminoethyl)glycine, equimolecular amounts of the two anhydrides were refluxed in dry $PhNO_2$.

The CBZ-protecting group was removed with 40% HBr in glacial AcOH. After standing overnight the resulting hydrobromide was precipitated with dry Et_2O. The free base was obtained by passing the H_2O solution of the polymer hydrobromide through an Amberlite IRA 400 ion exchanger. The eluate, which gave positive reaction with ninhydrin and a negative Volhard test, was freeze-dried and the crude product, tested according to Abderhalden and Komm[11] and to Sela and Berger,[10] showed no positive reaction for usual secondary products. The polymer was then fractionated through Sephadex G 10 and each fraction was separately freeze-dried. $\overline{DP}$ were determined by osmometric methods (H_2O solution).

Biological Testing.—Antibacterial activity against *Escherichia coli* was determined by means of an aerated thermoturbidimeter[5] by comparing the activity of the new products with that of polyornithine. A synthetic medium was used in growth experiments.[3f] Different concentrations of each substance were used in order to have, when possible, comparable lag periods. Results

(9) M. Bergmann, L. Zervas, and W. F. Ross, *J. Biol. Chem.*, **111**, 245 (1935).

(10) M. Sela and A. Berger, *J. Amer. Chem. Soc.*, **77**, 1893 (1955).

(11) E. Abderhalden and E. Komm, *Z. Physiol. Chem.*, **139**, 181 (1924).

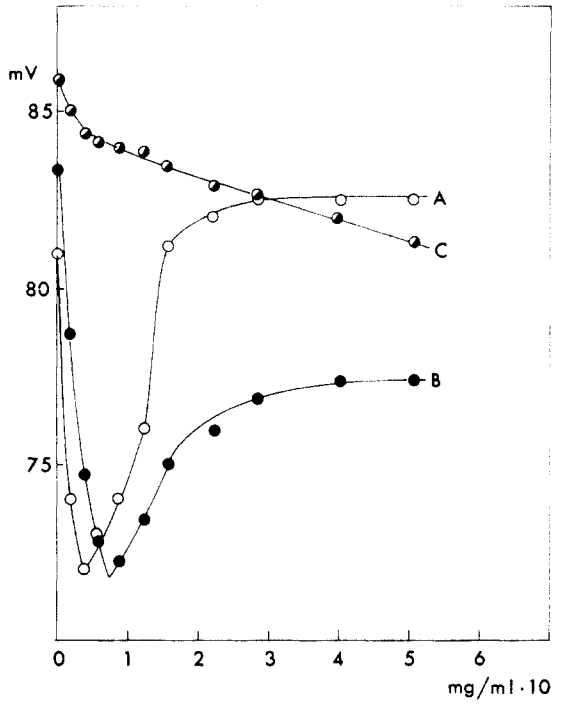


Figure 2.—Electromotive force recorded in a suspension of *E. coli* (1×10^{12} viable cells/ml) plus 1×10^{-4} *M* KCl as a function of the increasing concentration of *N*-(β-aminoethyl)-polyglycine ($\overline{DP}$ = 19 in plot A; $\overline{DP}$ = 6 in plot B) and of the monomer (plot C).

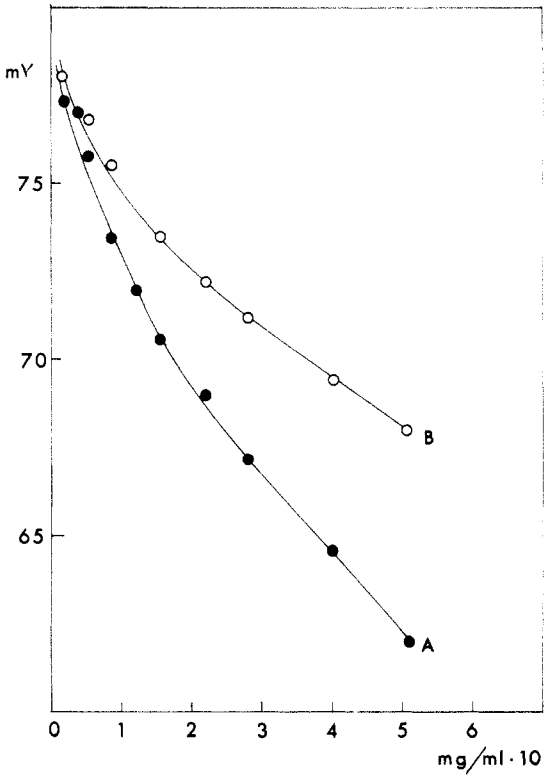


Figure 3.—Electromotive force recorded in a solution of the copolymer of ornithine-*N*-(β-aminoethyl)glycine ($\overline{DP}$ = 5) in 1×10^{-4} *M* KCl, *vs.* the copolymer concentration; plot A (●——●). Electromotive force recorded in a suspension of *E. coli* (1×10^{12} viable cells/ml) plus 1×10^{-4} *M* KCl as a function of the increasing concentration of the copolymer; plot B (○——○).

are summarized in Table I. The first reading (zero time) was done immediately after the addition of the test compounds.

E. coli cultures (5×10^6 viable cells per ml at zero time) were aerated with sterile air (8 l./hr) at 37° ± 0.1. The lag period is the interval in minutes between zero time and the first detectable increase of turbidity. The growth period is the length of the logarithmic phase.

Physicochemical Measurements.—Potentiometric measurements were made as described in a previous paper.[4b] In the present studies *E. coli* that had been cultured on nutrient agar were washed twice and suspended in 1×10^{-4} *M* KCl to a concentration of 1×10^{12} viable cells per ml. Calibration curves for the polyamino acids in 1×10^{-4} *M* KCl were obtained by both stepwise dilution and concentration of the solution while recording the electromotive force. The calibration curves (Figure 1) show the contribution to the electromotive force of the polymers.

The titration of *E. coli* suspension in 1×10^{-4} *M* KCl with the polymer in 1×10^{-4} KCl is given in Figure 2. The pH was 7.2 and there was no significant change during the titration. The titration curves that were obtained may be explained by the presumed interaction that takes place between the positively charged peptides and the negatively charged bacterial surface,[8b] which alters the charge distribution on the latter. Thus the mean ionic activity of the solution changes and the resulting curves differ completely from the calibration curve of each component. The formation of a complex between *E. coli* and the polymer is accompanied by an increase in the mean ionic activity in solution as indicated by the fall in the measured electromotive force. This may be explained by a release of K^+ from binding sites on the bacterial surface by the polymer, *i.e.*, a polymer–K^+ exchange on the surface. This process continues until a critical amount of polymer is added. Further addition of the polymer produces a sharp reversal of the curve followed by a situation of equilibrium. At this point an increase in the amount of peptide no longer contributes to the electromotive force; the mixture behaves like a solution of peptide that contains a suspension of stabilized *E. coli*–polymer complexes.

The titration curve of the monomer (Figure 2C) indicates that no interaction occurs with *E. coli*. The same electrochemical behavior was observed with other basic amino acids including L-lysine.

The calibration and titration curves of the copolymer (Figure 3) do not differ in trend indicating that no electrostatic interaction takes place between components despite the presence of basic residues in the copolymer. The same result was obtained when Poly-IIb was used as a titrant. Initially we thought that the presence of charges with opposite sign would insure electrostatic interaction between components of our system. However, the results obtained with the copolymer and Poly-IIb indicate that specific nonelectrostatic forces must be of considerable magnitude in determining the extent of the *E. coli*–peptide interaction, since they are capable, in the experimental conditions given (Figure 3B), of inhibiting the interaction itself. This is a new aspect of the complex of specific forces pointed out by Katchalski, *et al.*,[3c] which, though obscure and unknown, do exist and contribute to a large part of the interaction. A comparison of the physicochemical measurements and the biological tests of the present experiments suggests that (a) electrostatic interaction is accompanied by biological activity and (b) the presence of basic residues in a peptide is not sufficient in itself to insure electrostatic interaction, at least in the case of the polymers synthesized.