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The Synthesis and Circular Dichroism of a Series of Peptides Possessing the Structure (L-Tyrosyl-L-alanyl-L-glutamyl)$_n$

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A series of peptides possessing the structure (L-Tyr-L-Ala-L-Glu)$_n$, with n = 1, 2, 3, 4, 7, 9, and 13 was synthesized. The oligomers up to n = 4 were prepared by stepwise synthesis using the *N*-hydroxysuccinimide ester of the tripeptide derivative for the elongation of the chain. The oligomers with n = 7, 9, 13 were prepared by a polycondensation technique, followed by gel filtration.

The circular dichroism spectra of the above oligopeptides were measured in the wavelength range 200–330 nm in a solution of 0.15 M sodium chloride – 0.02 M sodium phosphate, pH 7.4, conditions at which the high molecular weight polytripeptide (Tyr-Ala-Glu)$_n$ exists in a helical conformation. The circular dichroic spectrum of the high molecular weight polymer exhibits two negative ellipticity bands: an intense band at 220 nm ($[\theta]_{max}$ = 8700) and a weak band at 273 nm ($[\theta]_{max}$ = 360). The tripeptide (n = 1) exhibits a positive ellipticity band at 227 nm ($[\theta]_{max}$ = 4000) and a broad, positive, weak band at 270 nm ($[\theta]_{max}$ = 115). The other oligopeptides (n = 2 to 13) exhibit positive bands in the 227 nm region, with ellipticity values that decrease as the degree of polymerization increases. Except for (Tyr-Ala-Glu)$_{13}$, which has a negative peak centered at 216 nm, circular dichroic spectra of the other oligomers (n = 1 to 9) in the 200–210 nm region resemble that of random coils. In the 260–290 nm region, the circular dichroic curves of the oligopeptides gradually approach the 272 nm band of the polypeptide (Tyr-Ala-Glu)$_n$, as the degree of polymerization increases. Under physiological conditions, only the (Tyr-Ala-Glu)$_{13}$ shows an indication of helical content.

The high molecular weight polytripeptide with the repeating sequence L-tyrosyl-L-alanyl-L-glutamyl was synthesized and characterized by Ramachandran *et al.* [1–3]. This polymer, which has been shown to possess an α-helical conformation under physiological conditions, was used by us for an investigation of the nature of antigenic determinants in macromolecules [3–6]. In connection with this study, which is described in the accompanying paper [4] and others to follow [5,6], we have synthesized a series of peptides of the structure (Tyr-Ala-Glu)$_n$, where n = 1, 2, 3, 4, 7, 9, 13. The peptides (Tyr-Ala-Glu)$_n$, with n = 1 to 4 were prepared by a stepwise synthesis using the succinimide ester of *N*,*O*-dibenzyloxycarbonyl-L-tyrosyl-L-alanyl-γ-benzyl-L-glutamate as a unit of elongation. After removal of the blocking groups the free peptides were fractionated by means of gel filtration, resulting in essentially homogeneous products. The peptides (Tyr-Ala-Glu)$_n$, with n = 7, 9, 13, were obtained as products of polycondensation of the activated tripeptide, following the synthesis of Ramachandran *et al.* [1,2]. The three fractions obtained on gel filtration had average molecular weights of 2656, 3200 and 4926, corresponding to the average n values of 7, 9 and 13.

In connection with the immunological investigations it was of interest to assess the polypeptide length required for the onset of helicity in the series of peptides. Previously reported studies dealing with the problem of the critical size for helix formation in polypeptides were based on far ultraviolet absorption and optical rotatory dispersion measurements [7–14]. It was demonstrated that the lowest polymeric size for the onset of helicity in non-aqueous solvents is an octapeptide of γ-methyl glutamate [15], nonaalanine [16] or undecapeptide of β-methyl aspartate [17]. When circular dichroism was recently used by Goodman *et al.* [18] to investigate the same problem, the data indicated that a helical structure can initially be formed already with the heptapeptide of γ-ethyl glutamate.

In the present study, the circular dichroic spectra of the (Tyr-Ala-Glu)$_n$ series of oligopeptides were measured and compared to those of the high molecular

Unusual Abbreviations. Oligopeptides (Tyr-Ala-Glu)$_n$, peptides containing up to 13 residues each of L-tyrosine, L-alanine and L-glutamic acid in regular alternating sequence; polypeptide (Tyr-Ala-Glu)$_n$, a helical polymer of the regular alternating tripeptide Tyr-Ala-Glu with n approx. 200; polypeptide (Tyr,Ala,Glu)$_n$, random copolymer of L-tyrosine, L-alanine and L-glutamic acid.

weight helical copolymer $(Tyr\text{-}Ala\text{-}Glu)_n$, as well as to a high molecular weight random copolymer of L-tyrosine, L-alanine and L-glutamic acid, $(Tyr,Ala,Glu)_n$. All measurements were performed in 0.15 M sodium chloride—0.02 M phosphate buffer, pH 7.4. In this medium the polypeptide $(Tyr\text{-}Ala\text{-}Glu)_n$ exists in its helical conformation.

MATERIALS AND METHODS

Syntheses

Melting points were determined with a Buchi apparatus and are uncorrected. Paper chromatography was carried out on Whatman No. 1 filter paper at room temperature in 1-butanol—acetic acid—water (4:1:4, by vol.). High voltage electrophoresis at pH 1.9 was carried out as described [19]. Peptide spots were located by ninhydrin. The equivalent weights of peptide derivatives containing free carboxylic groups were determined by non-aqueous titration with 0.05 N methanolic sodium methoxide [20], using thymol blue as indicator. Amino acid composition of acid hydrolysates of peptides were quantitatively determined according to Spackman *et al.* [21] on an automatic amino acid analyzer Beckman-Spinco Model 120 B. All amino acids used in this study are of the L configuration.

t-*Butyloxycarbonyl alanine succinimide ester (I)* was prepared according to Anderson [22]. M. p. 146 to 148° C (reported 143—144° C). Anal. calc.: N, 9.8; found: N, 9.8.

t-*Butyloxycarbonyl alanyl-γ-benzyl-glutamate (II).* γ-Benzyl glutamate (4.74 g, 20 mmole) was dissolved in 150 ml boiling water. Cooling of the solution resulted in the formation of a fine floccular suspension of the compound. To the γ-benzyl glutamate suspension, sodium bicarbonate (1.68 g, 20 mmoles) and a solution of I (5.72 g, 20 mmoles) in 100 ml dioxane were added, followed by the addition of more sodium bicarbonate (1.68 g, 20 mmoles). The clear solution was kept at 4° C overnight. The dioxane was removed *in vacuo* and the aqueous solution was concentrated to 100 ml, cooled in ice and adjusted to pH 3 with HCl. The oily product which separated was washed with 0.01 N HCl (4×40 ml) and water (4×50 ml). The oily precipitate was dissolved in benzene, dried over anhydrous sodium sulphate and was precipitated by petroleum ether. Reprecipitation from benzene with petroleum ether yielded 5.5 g (67%). M. p. 89° C. Equivalent weight: 407. Anal. calc. for $C_{20}H_{28}N_2O_7$ (408.4): C, 58.8; H, 6.91; N, 6.86. Found: C, 58.8; H, 6.77; N, 6.92.

Alanyl-γ-benzyl-glutamate hydrochloride (III). Compound II (3 g, 7.3 mmoles) was dissolved in 40 ml 3.5 N HCl in dioxane and kept at room temperature for 30 min. The dioxane was then removed *in vacuo* and ether was added. The viscous product which precipitated solidified after several washings with ether. Yield, 2.2 g (87%). III was found to be homogeneous by high voltage paper electrophoresis at pH 1.9, and by paper chromatography in butanol—acetic acid—water.

N,O-*Dibenzyloxycarbonyl-tyrosine succinimide ester.* *N,O*-Dibenzyloxycarbonyl-tyrosine [23] (9 g, 20 mmoles) and *N*-hydroxysuccinimide (2.3 g, 20 mmoles) were dissolved in 40 ml dioxane— ethyl acetate (1:1). After cooling the solution to 0° C, *N,N'*-dicyclohexylcarbodiimide (4.12 g, 20 mmoles) was added. The reaction mixture was kept overnight at 4° C. The dicyclohexylurea formed was removed, and the filtrate was evaporated to yield a solid product which was recrystallized from isopropanol—dioxane (3:1). Yield 7.4 g (68%); m. p., 150° C. Anal. calc.: N, 5.1; found: 4.8.

N,O-*Dibenzyloxycarbonyl-tyrosyl-alanyl-γ-benzyl-glutamate (IV).* A solution of III (3.9 g, 11.3 mmoles) and sodium bicarbonate (1.42 g, 17 mmoles) in 35 ml water was added gradually to a solution of *N,O*-dibenzyloxycarbonyltyrosine succinimide ester (6.2 g, 11.3 mmoles) in 70 ml dioxane, followed by the addition of more sodium bicarbonate (0.47 g, 5.6 mmoles). The reaction mixture was kept overnight at room temperature and then it was poured into 400 ml water containing HCl (40 mmoles). The oily product which precipitated solidified after 30 min and was washed with 0.01 N HCl and water. The material was recrystallized from benzene to yield 6.4 g (76%); m. p., 114° C. Equivalent weight found by tritation with anhydrous sodium methoxide, 735; calcd.: 739.

N,O-*Dibenzyloxycarbonyl-tyrosyl-alanyl-γ-benzyl-glutamate succinimide ester (V).* A solution of IV (6.3 g, 8.5 mmoles) and *N*-hydroxysuccinimide (1.96 g, 17 mmoles) in 32 ml dioxane—ethyl acetate (1:1) was cooled to 0° C and dicyclohexylcarbodiimide (1.76 g, 8.5 mmoles) was added. The reaction mixture was kept overnight at 4° C. After removal of the dicyclohexylurea the filtrate was evaporated to yield an oily residue which was dissolved in 10 ml chloroform and the product was precipitated upon addition of ether. Yield, 6.2 g (87%). Anal. calc.: N, 6.7; found: N, 6.6.

N,O-*Dibenzyloxycarbonyl-tyrosyl-alanyl-glutamic acid.* A solution of alanyl-glutamic acid (2.17 g, 9.6 mmoles) and sodium bicarbonate (2.42 g, 28.8 mmoles) in 25 ml water was added to a solution of *N,O*-dibenzyloxycarbonyltyrosine succinimide ester (5.25 g, 9.6 mmoles) in 25 ml dioxane. The reaction mixture was kept overnight at room temperature and then the dioxane was removed *in vacuo*. The viscous residue was dissolved in 200 ml water, and the product which was precipitated by adding HCl (33 mmole) was washed with 0.01 N HCl and water. After recrystallization from ethyl acetate the yield was 4 g (64%); m. p. 140° C. Equivalent weight found by

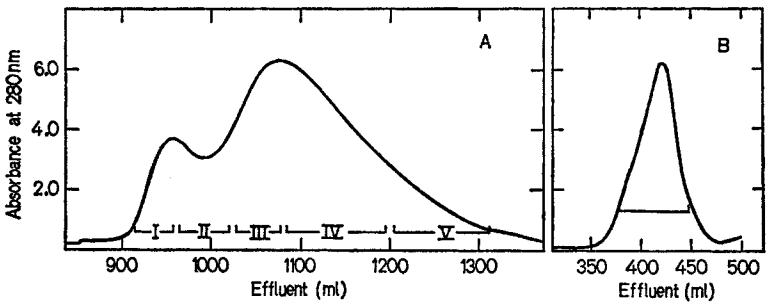


Fig. 1. (A) *Chromatography of the hexapeptide (Tyr-Ala-Glu)$_2$ on a column (1.7×200 cm) of Sephadex G-10 in 0.01 N HCl at 25° C.* The flow rate was 54 ml per hour. According to high voltage electrophoresis at pH 1.9, fraction I contains 98% Tyr-Ala-Glu, fraction III contains 70% Tyr-Ala-Glu and 30% (Tyr-Ala-Glu)$_2$ and fractions III—V contains more than 90% (Tyr-Ala-Glu)$_2$ (mole/mole). (B) *Rechromatography of fractions III—V on a column (1.7×200 cm) of Sephadex G-15 in 0.05 M NH_4HCO_3, pH 8.6 at 25° C.* The flow rate was 60 ml/h. According to high voltage electrophoresis at pH 1.9 the hexapeptide was > 98% pure

Table. *Amino acid analysis of (Tyr-Ala-Glu)$_n$ peptides*

Peptide	Tyr	Ala	Glu
		molar ratio	
Tyr-Ala-Glu	0.93	0.97	1.00
(Tyr-Ala-Glu)$_2$	1.01	1.02	1.00
(Tyr-Ala-Glu)$_3$	0.92	1.00	1.00
(Tyr-Ala-Glu)$_4$	0.97	1.01	1.00

titration with anhydrous sodium methoxide, 326; calc.: 324.5. Anal. calc.: N, 6.48; found: N, 6.44.

Tyrosyl-alanyl-glutamic acid. *N,O*-Dibenzyloxycarbonyl-tyrosyl-alanylglutamic acid (6.1 g, 9.4 mmoles) was dissolved in 100 ml acetic acid. A suspension of palladium on charcoal (0.3g, 10% catalyst) in 10 ml water was added and the hydrogenolysis was carried out at room temperature and at a pressure of 3 atm for 24 h. The catalyst was filtered off, and the filtrate was evaporated *in vacuo* to yield an oily product which solidified after the addition of ethanol (100 ml) and ether (100 ml). Yield: 3.12 g (87%). The peptide was found to be homogeneous in high voltage paper electrophoresis at pH 1.9, and paper chromatography in butanol—acetic acid—water. Amino acid analysis of an acid hydrolysate of the peptide is given in the Table.

N,O-*Dibenzyloxycarbonyl-tyrosyl-alanyl-γ-benzyl-glutamyl-tyrosyl-alanylglutamic acid (VI$_2$).* A solution of tyrosyl-alanyl-glutamic acid (1.14 g, 3 mmoles) and sodium bicarbonate (0.75 g, 9 mmole) in 30 ml water was added gradually to a solution of V (2.51 g, 3 mmoles) in 30 ml dioxane, and was followed by vigorous shaking. The solution became clear after 5 min and a solid product precipitated after 30 min. The reaction mixture was kept overnight at room temperature and then water (150 ml) and HCl (12 mmoles) were added. The precipitate formed was washed with 0.01 N HCl and water. Yield: 3.05 g (92%). Equivalent weight found by titration with anhydrous sodium methoxide, 560; calc.: 551.

Tyrosyl-alanyl-glutamyl-tyrosyl-alanyl-glutamic acid. VI$_2$ (3.5 g, 3.16 mmoles) was subjected to hydrogenolysis as described above. Yield: 2.25 g (96%). Upon high voltage electrophoresis at pH 1.9, two spots were detected with ninhydrin: 85—90% the hexapeptide and 10—15% of the tripeptide (mole/mole). The hexapeptide was purified on a column of Sephadex G-10 in 0.01 N HCl, and then on a column of Sephadex G-15 in 0.05 M NH_4HCO_3, pH 8.6 (Fig. 1). The product obtained gave one spot with ninhydrin in high voltage paper electrophoresis at pH 1.9 (> 98% pure). Amino acid analysis of an acid hydrolysate of the hexapeptide is given in the Table.

N,O-*Dibenzyloxycarbonyl-tyrosyl-alanyl-γ-benzyl-glutamyl-tyrosyl-alanyl-glutamyl-tyrosyl-alanyl-glutamic acid (VI$_3$).* (Tyr-Ala-Glu)$_2$ (0.95 g, 1.27 mmoles) was coupled to V (1.17 g, 1.4 mmoles) in the same manner described for VI$_2$. Yield: 1.42 g (76%). Equivalent weight found by titration with anhydrous sodium methoxide, 520; calc., 488.

(Tyr-Ala-Glu)$_3$. VI$_3$ (1.42 g, 0.97 mmole) was subjected to hydrogenolysis as described above. Yield: 0.9 g (87%). Upon high voltage paper electrophoresis at pH 1.9 three spots were detected with ninhydrin: 70% of the nonapeptide, 2% of the hexapeptide and 28% of the tripeptide (mole/mole). The nonapeptide was purified on a column of Sephadex G-10 in 0.01 N HCl and then on a column of Sephadex G-15 in 0.05 M NH_4HCO_3, pH 8.6 (Fig. 2). The product obtained gave one ninhydrin positive spot in high voltage electrophoresis at pH 1.9 (> 98% pure). Amino acid analysis of an acid hydrolysate of the nonapeptide is given in the Table.

N,O-*Dibenzyloxycarbonyl-tyrosyl-alanyl-γ-benzyl-glutamyl-tyrosyl-alanyl-glutamyl-tyrosyl-alanyl-glutamyl-tyrosyl-alanyl-glutamic acid (VI$_4$).* (Tyr-Ala-Glu)$_3$ (0.36 g, 0.32 mmoles) was coupled to V (0.52 g, 0.62 mmoles) in the same manner as described for VI$_2$.

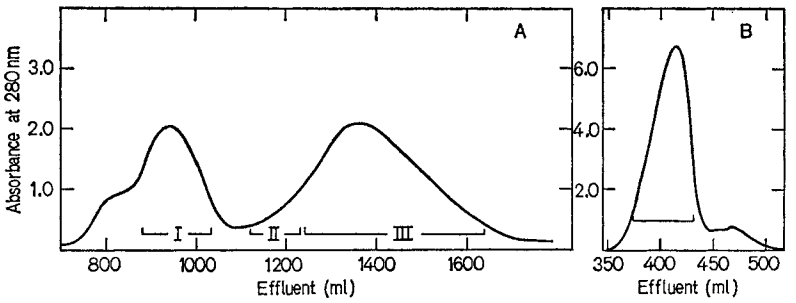


Fig. 2. (A) *Chromatography of the nonapeptide (Tyr-Ala-Glu)$_3$ on a column (1.7×200 cm) of Sephadex G-10 in 0.01 N HCl at 25° C.* According to high voltage electrophoresis at pH 1.9, fraction I contains 85% Tyr-Ala-Glu, fraction II contains 85% (Tyr-Ala-Glu)$_3$ and fraction III contains 95% (Tyr-Ala-Glu)$_3$ (mole/mole). (B) *Rechromatography of fraction III on a column (1.7×200 cm) of Sephadex G-15.* According to high voltage electrophoresis at pH 1.9 the nonapeptide was > 98%

(Tyr-Ala-Glu)$_4$. Compound VI$_4$ (0.8 g) was dissolved by boiling in 40 ml acetic acid (the material precipitated after cooling) and a suspension of palladium on charcoal (0.1 g, 10% catalyst) in 10 ml water was added. Hydrogenolysis was carried out at room temperature and at a pressure of 3 atm during 18 h. The filtrate obtained after removal of the catalyst was evaporated *in vacuo* to yield an oily product which solidified after adding ethanol and ether to yield 0.4 g. Upon high voltage electrophoresis at pH 1.9 three spots were detected with ninhydrin: 60% of the dodecapeptide, 5% of the nonapeptide, and 35% of the tripeptide (mole/mole). Upon chromatography on a Sephadex G-10 column in 0.01 N HCl, the tripeptide was eluted as expected, whereas only a small portion of the dodecapeptide was eluted, not as one peak but in a large volume of the eluate. It seems, therefore, that the dodecapeptide was absorbed on the Sephadex and most of it was lost. The small quantity obtained was lyophilized and rechromatographed on Sephadex G-15 column in 0.05 M NH_4HCO_3, pH 8.6. One peak was obtained which contained the dodecapeptide. One spot was detected with ninhydrin upon high voltage electrophoresis at pH 1.9 (> 97% pure). Amino acid analysis of an acid hydrolysate of the peptide is given in the Table.

Random Copolymer of L-tyrosine, L-alanine and L-glutamic acid. The polypeptide (Tyr,Ala,Glu)$_n$ was synthesized as described previously [24], and in a paper [6] to follow. It had an average molecular weight of 15000 and a residue molar ratio of Tyr-Ala-Glu of 1.0:1.5:1.5.

Methods

Molecular Weight Determination. Ultracentrifugation runs were performed at 20° C in a Spinco ultracentrifuge model E (Beckman Instruments). The molecular weights were determined by the Yphantis short column sedimentation equilibrium method [25]. The measurements were made in 0.15 M sodium chloride, 0.02 M phosphate buffer, pH 7.4, and peptide at 1% concentration.

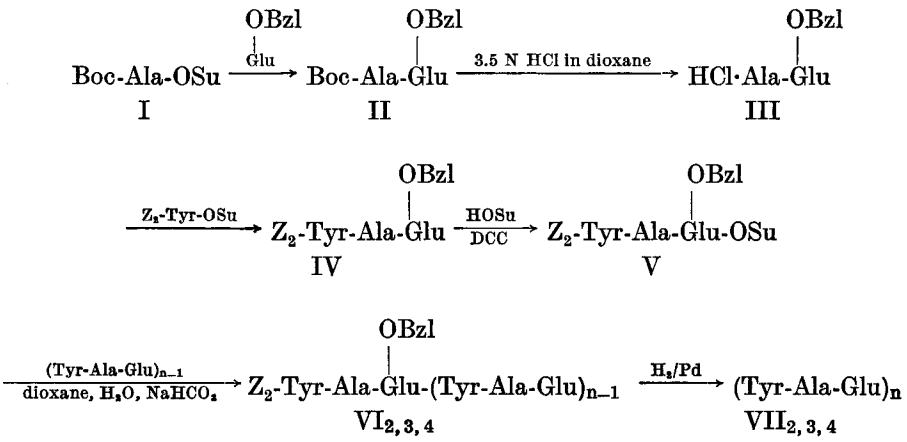
Circular Dichroism Measurements. Circular dichroic spectra were measured in the wavelength range 200—330 nm at 27° C in a Cary Model 60 recording spectropolarimeter with circular dichroic attachment. Samples were in 0.15 M sodium chloride —0.02 M sodium phosphate, pH 7.4. Concentrations of the peptide solutions were determined from quantitative amino acid analysis. The absorbance of solutions did not exceed 1.9 absorbance units. The path length was varied from 1 cm to 1 mm. The circular dichroism data are expressed in terms of the mean residue ellipticity $[\theta]$ defined as $[\theta]_\lambda^{27} = M\,\theta/10\,cl$, with units of deg (cm)^2 per decimole, where θ is the observed ellipticity in degrees, M is the mean residue weight [121 for the polypeptide (Tyr-Ala-Glu)$_n$ and 116 for the random polypeptide (Tyr,Ala,Glu)$_n$], l is the path length in cm and c is the concentration in g/ml. Molecular ellipticity was not corrected for the refractive index of the solvent [26].

RESULTS AND DISCUSSION

Synthesis of the Oligopeptides (Tyr-Ala-Glu)$_n$, n = 1, 2, 3, 4

All amino acids are of L-configuration. The oligopeptides were synthesized according to the following scheme (see page 305), where Boc = *t*-butyloxycarbonyl, Bzl = benzyl, OSu = succinimide ester, HOSu = *N*-hydroxysuccinimide, DCC = *N,N'*-dicyclohexylcarbodiimide and Z_2 = *N,O*-dibenzyloxycarbonyl.

The "*N*-hydroxysuccinimide" esters [22] were used in the present study for the stepwise synthesis of the tripeptides as well as for the condensation reaction between the tripeptide derivative and the free peptide (Tyr-Ala-Glu)$_{n-1}$. The succinimide esters were found to be suitable for synthesis of the above oligopeptides as the easily crystallized compounds



Scheme

are highly reactive towards amines in aqueous solutions. *t*-Butyloxycarbonylalanine was coupled to *N*-hydroxysuccinimide with dicyclohexylcarbodiimide according to Anderson *et al.* [22] to yield I. Reaction of I with γ-benzyl glutamate gave *N*-*t*-butyloxycarbonyl-alanyl-γ-benzyl glutamate (II). The *t*-butyloxycarbonyl group was used to block the amino terminus of II in this step because it can be selectively removed by HCl in dioxane (III). The benzyloxycarbonyl was used as a blocking group in the synthesis of IV and in the final step ($VI_{2,3,4}$) all blocking groups were removed by catalytic hydrogenolysis, yielding the free peptides ($VII_{2,3,4}$).

In order to prevent possible racemization of the glutamic acid residue in compound IV, coupling to *N*-hydroxysuccinimide with the aid of dicyclohexylcarbodiimide was carried out at 0° C and under acidic conditions. For this purpose two equivalents of *N*-hydroxysuccinimide per 1 equivalent of the other reactants were used [27]. It should be noted that the carboxy-terminal amino acid in the activated tripeptide, the glutamic acid, is quite resistant to racemization.

The active ester V is soluble in dioxane and tends to precipitate out as oily droplets upon addition of water. The aqueous solution of the free peptide was, therefore, added gradually to the dioxane solution of the active ester (V) with vigorous shaking. The oily droplets dissolved after reaction, and the mixture became clear. Addition of water, followed by adjustment of the pH to pH 3, resulted in the precipitation of both VI and of unreacted IV. The unreacted water soluble $(Tyr\text{-}Ala\text{-}Glu)_{n-1}$, *N*-hydroxysuccinimide, and sodium chloride were removed by filtration. Catalytic hydrogenolysis of the product yielded a mixture of unreacted tripeptide and the product $(Tyr\text{-}Ala\text{-}Glu)_{n-1}$. The oligopeptide was purified by chromatography on Sephadex columns.

Separation of the peptides on Sephadex (G-15 or G-50) columns in 0.05 M NH_4HCO_3 was ineffective. It seems that the Sephadex carries some negatively charged carboxylic groups, and at pH 8.6 it repulses the $(Tyr\text{-}Ala\text{-}Glu)_n$ molecules which are also negatively charged. The tri- or hexapeptide does not penetrate the Sephadex particles and its elution pattern is similar to that of the nonapeptide. A satisfactory separation between the peptides was obtained using Sephadex G-10 in 0.01 N HCl, where carboxylic groups are not ionized. Unexpectedly, the order of peptides being eluted was reversed: the tripeptide was first to be eluted, then the hexa- and finally the nonapeptide (Figs. 1, 2). In this case, phenolic groups of tyrosine may cause absorption of the peptides on the Sephadex (the tripeptide Tyr-Ala-Glu, molecular weight 381, is eluted with 950 ml 0.01 N HCl, whereas a pentaalanine of molecular weight 373 is eluted in the same column conditions with 380 ml 0.01 N HCl). Therefore, the separation of the $(Tyr\text{-}Ala\text{-}Glu)_n$ peptide is achieved because of an adsorption rather than a sieving process. The higher the value of n, the greater the adsorption factor, so that the dodecapeptide $(Tyr\text{-}Ala\text{-}Glu)_4$ could be eluted from the column only after using a large volume of eluate.

High Voltage Paper Electrophoretic Separation at pH 1.9 of $(Tyr\text{-}Ala\text{-}Glu)_n$ Peptides, $n = 1$ to 4

At pH 1.9 the carboxylic groups in the peptides are not ionized. The peptides, therefore, will move towards the cathode due to the positive charge of the amino terminus, and the mobility will be proportional to the ratio between the charge and the molecular weight. As seen in Fig. 3, satisfactory separation of all peptides was achieved.

Synthesis of the Oligopeptides (Tyr-Ala-Glu)$_n$, $n = 7, 9, 13$

The succinimide ester of *O*-benzyl-L-tyrosyl-L-alanyl-γ-benzyl-L-glutamate was polymerized according to the procedure of Ramachandran *et al.* [1,2]. The benzyl groups were removed by means of hydrogen bromide and the resulting free polypeptide was gel filtered on Sephadex G-50 in 0.05 M ammonium acetate, pH 8.0. The eluted material was pooled into three fractions, A, B and C, possessing the average molecular weights of 4936, 3200 and 2656, respectively, as determined by ultracentrifugal analysis.

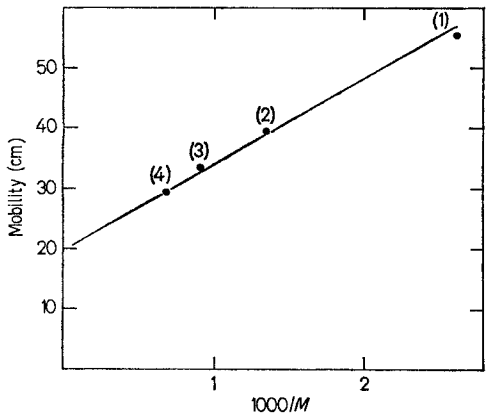


Fig. 3. *Electrophoretic separation of the peptides (1) Tyr-Ala-Glu, (2) (Tyr-Ala-Glu)$_2$, (3) (Tyr-Ala-Glu)$_3$ and (4) (Tyr-Ala-Glu)$_4$ in formic acid buffer pH 1.9, after 4 h at 30 V/cm. M*, molecular weight

Circular Dichroism Studies

The circular dichroism technique has been applied to conformational analysis of polypeptides [28,29] and proteins [29,30]. A characteristic circular dichroic spectrum was demonstrated either for the helical form or for the random coil structure of a number of homopolymers and copolymers [29]. Where tyrosine residues are involved, a different spectrum has been observed [31] most likely due to side chain transitions of the aromatic groups.

In the present study the circular dichroism spectra of the polypeptides (Tyr-Ala-Glu)$_n$ and (Tyr,Ala, Glu)$_n$ and the oligopeptides (Tyr-Ala-Glu)$_n$ (n = 1, 2, 3, 4, 7, 9, 13) were measured in the wavelength range 200—330 nm under conditions (0.15 M sodium chloride—0.02 M sodium phosphate, pH 7.4) at which the polypeptide (Tyr-Ala-Glu)$_n$ exists in its helical conformation [1,2] (Fig. 4). The circular dichroism spectrum of the helical polypeptide (Tyr-Ala-Glu)$_n$ is composed of two negative ellipticity

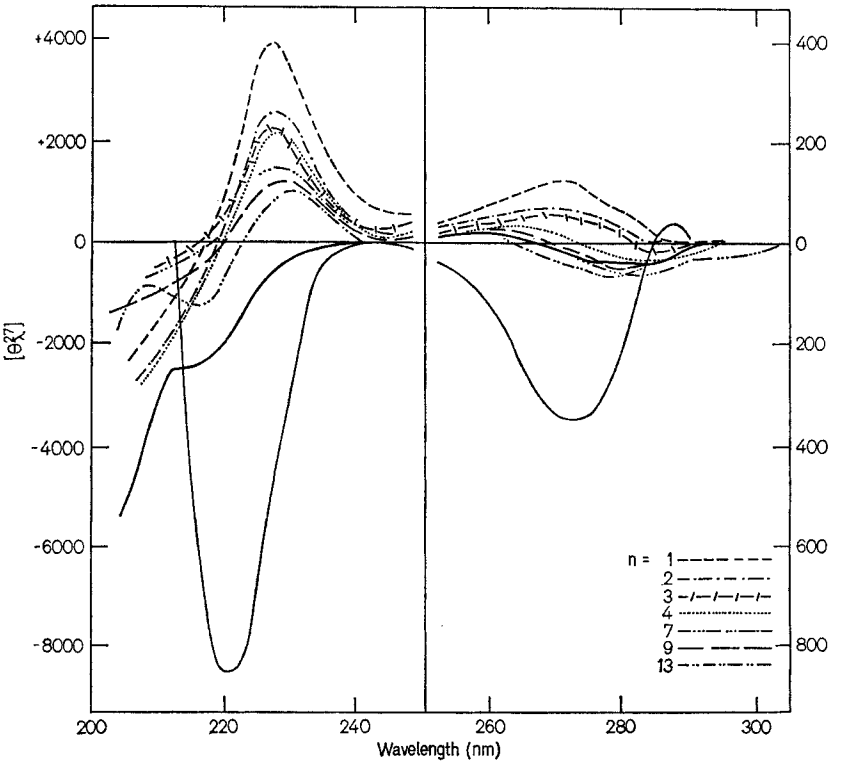


Fig. 4. *Circular dichroic spectra of the oligopeptides (Tyr-Ala-Glu)$_n$ where n = 1, 2, 3, 4, 7, 9 or 13, the random polypeptide (Tyr,Ala,Glu)$_n$ and the high molecular weight ordered polypeptide (Tyr-Ala-Glu)$_n$ (n = 200 approx.), in 0.15 M sodium chloride—0.02 M sodium phosphate buffer, pH 7.4*

bands centered at 220 nm ($[\theta]_{max} = 8700$) and 273 nm ($[\theta]_{max} = 360$), and a very weak positive band at 287 nm ($[\theta]_{max} = 40$). The 220 nm band of the polypeptide $(Tyr\text{-}Ala\text{-}Glu)_n$ is most likely analogous to the 224 nm band of poly-L-tyrosine [31].

The circular dichroic spectrum of the polypeptide $(Tyr\text{-}Ala\text{-}Glu)_n$ in the wavelength range 230—300 nm is, however, completely different from that observed for helical poly-L-tyrosine. Differences in the optical properties of these two polypeptides may be due to differences in side chain interactions. The conformational analysis of the polypeptide $(Tyr\text{-}Ala\text{-}Glu)_n$ made by Ramachandran *et al.* [1,2] indicates that the helical structure of this polypeptide is stabilized by side chain interactions. The phenolic groups of tyrosine residues form hydrogen bonds with carboxylic groups of glutamic acid residues. The polypeptide $(Tyr\text{-}Ala\text{-}Glu)_n$ can, therefore, assume a helical conformation at neutral pH in the presence of 0.15 M sodium chloride, whereas poly-L-tyrosine is helical only at pH values above pH 11.0.

The tripeptide Tyr-Ala-Glu exhibits an intense positive ellipticity band at 227 nm ($[\theta]_{max} = 4000$) and a broad positive weak band at 270 nm ($[\theta]_{max} = 115$) (Fig. 4). The other oligopeptides (n = 2, 3, 4, 7, 9, 13) exhibit positive bands in the 227 nm region, with ellipticity values that decrease as the n value of the oligopeptide increases. In the 200—210 nm region the curves show a trend towards the 195 to 200 nm negative band characteristic of the random coil structure [29], except for the curve of $(Tyr\text{-}Ala\text{-}Glu)_{13}$ which has a negative peak centered at 216 nm, possibly reflecting the existence of an incipient helical species or of partially helical molecules in the solution. In the 260—290 nm region, the curves of the oligopeptides are gradually approaching the 273 nm band of the polypeptide $(Tyr\text{-}Ala\text{-}Glu)_n$.

It is rather surprising that a peptide as long as the $(Tyr\text{-}Ala\text{-}Glu)_{13}$ (39 amino acid residues on the average) is required for the initial formation of a helical structure. Previous studies dealing with the problem of the critical size for helix formation indicated shorter peptide length, although the comparison may not be valid as the measurements were performed in non-aqueous solvents [15—17].

The gradual changes in the ellipticity bands of the oligopeptides may be due to end group effects. It was suggested by Brand *et al.* [32], Doty and Geiduschek [33] and Goodman *et al.* [34] that the end groups of the peptide solvate differently from internal residues. Thus, their contribution to the optical activity is different from internal residues. As the molecular weight of the peptide increases, the contribution of end group effects will decrease.

A $(Tyr,Ala,Glu)_n$ random polypeptide was already shown by Ramachandran *et al.* [2] to contain some helical structure (not more than 30% helicity). Similarly, the polypeptide $(Tyr,Ala,Glu)_n$ sample we used exhibited a circular dichroic spectrum in the 200—250 nm region, compatible with some helical content.

In conclusion, the approach used in this study for the stepwise synthesis of oligomers of tripeptides may be of general use for the preparation of ordered polymers of increasing size. In the $(L\text{-}Tyr\text{-}L\text{-}Ala\text{-}L\text{-}Glu)_n$ series discussed here, results of circular dichroism investigations show that the onset of significant helical content occurs, under physiological conditions, only for peptides containing as many as 39 amino acid residues.

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