Unidirectional Stability of $\alpha$-Helix. Theoretical Calculation and Attempt for Synthesis of Block Copolypeptides

Toshiya Kontani*, Ken Nishikawa,** Takayoshi Iio,*** Sho Takahashi,** and Tatsuo Ooi**

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The stability of $\alpha$-helical structure in the $N$-terminal half and the $C$-terminal half of the helix, which was constructed of 30 residues, was investigated when the distortion was brought about at the middle of the chain. Calculation was carried on two parameters, $\Delta \tau$ and $\Delta E$, which represent the difference of the $i$-th rotational angle of peptide plane around the $i$-th virtual bond ($C^\alpha_i-C^\alpha_{i+1}$) and that of the energy of the $i$-th residue between the distorted conformation and the standard $\alpha$-helix respectively. The results of the calculation showed that both of the two values were convergent to zero along the $C$-terminal direction, but amplified in the $N$-terminal direction. We attempted to synthesize $(Glu)_{20}(Ala)_{20}$ and $(Ala)_{20}(Glu)_{20}$, which would offer experimental evidences for the results of the calculation to be justified, with a general method of solid-phase peptide synthesis. It revealed, however, that the method was unable to afford the desired products, because the yield of $(Glu)_{n}$ and $(Ala)_{n}$ were gradually decreased with increasing $n$. The practical limit of $n$ was found to be approximately 10.

INTRODUCTION

Many sorts of synthetic polypeptides have been synthesized and their conformations have been studied widely. Generally speaking, various homopolypeptides show the helix-coil transition according to the change of experimental condition, for example, solvent, temperature, etc. This transition is considered to take place in the order as follows; certain positions in a helical chain are distorted to coil states, then the coil states propagate to both sides, i.e., the $N$-terminal direction and the $C$-terminal direction, to change the neighboring residues from helical states to coil. It is not certain, however, whether the distortions propagate to both sides equally since polypeptide chain is not symmetrical with respect to the chain direction. In fact, such asymmetry was found in real protein. We tried to reveal this asymmetric character both in a theoretical calculation and in an experiment.

We carried out a calculation for the case that a distortion was brought about at the middle of the $\alpha$-helix of a finite length of 30 residues and the effect to the vicinal residues in both sides along the chain was investigated. The calculation clarified that helical conformation was disrupted only in the $N$-terminal direction. As a next step, we planned an experiment to detect such asymmetry and simultaneously to confirm

* 紺谷俊也 : Department of Molecular Biology, Cancer Research Institute, Kanazawa University, Kanazawa.
** 西川 建, 高橋 譲, 大井亀夫 : Laboratory of Physical Chemistry of Enzyme, Institute for Chemical Research, Kyoto University, Uji, Kyoto.
*** 齋尾隆義, Institute of Physics, Faculty of Science, Nagoya University, Nagoya.
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the result of the calculation. We attempted to synthesize two block copolypeptides, (Glu)$_{20}$(Ala)$_{20}$ and (Ala)$_{20}$(Glu)$_{20}$ which were considered to be long enough to form α-helix and had reverse amino acid sequences to one another, since the two polymers were expected to show different behaviors in a helix-coil transition.

THEORETICAL SECTION

Calculation Procedure In this report, we are only concerned with a particular case that all the C$\alpha$ atoms of the α-helical polypeptide are held fixed. Thereby, only a freedom remained in the backbone conformation is the rotation of the peptide group around the virtual bond connecting two successive C$\alpha$ atoms. Adopting the fixed values for all the bond lengths and bond angles, a rotation of one peptide group by a certain amount can not take place independently but is linked with rotations of the neighboring peptide groups. Thus, the distortion of a regular α-helix could be simply treated as one freedom system under the condition that all the C$\alpha$ atoms maintain the regular positions.

First, we must deduce the relationship between rotational angles of two successive peptide groups. The skeletal structure made by joining all the C$\alpha$ atoms is specified with two parameters: bond angle (θ) between the virtual bonds and dihedral angle (γ) between two planes C$_i^\alpha$—C$_i^\alpha$—C$_i+1$ and C$_i^\alpha$—C$_i+1$—C$_i+2$ (Fig. 1). In the present case, θ and γ are both the constant quantities because of the assumption mentioned above. The rotational angle of the i-th peptide plane (τ$_i$) is defined as the rotational position of the C$_i^\alpha$ atom measured clockwise from the standard plane of C$_{i-1}^\alpha$—C$_i^\alpha$—C$_{i+1}$ when C$_i^\alpha$ is on the standard plane and also on the same side as the C$_{i-1}^\alpha$ atom. There is a general relationship between the parameters of a virtual bond method and those of the usual real bond method (i.e., using φ$_i$ and ψ$_i$). The
The relation between the successive $z$'s can be led from the general equation (Eq. 11 of Ref. 3) with the elimination of $c_{pi}$ and $c_{i+1}$, i.e.,

$$\sin \xi \cos \eta \cos (\tau_i - \gamma) \cos \tau_{i+1} - \sin (\tau_i - \gamma) \sin \tau_{i+1}$$

$$+ \sin \theta \left[ \sin \xi \cos \gamma \cos (\tau_i - \gamma) - \cos \xi \sin \eta \cos \tau_{i+1} \right]$$

$$+ \cos \xi \cos \eta \cos \theta - \cos \alpha = 0 \quad (1)$$

where $\alpha$, $\xi$, and $\eta$ are the fixed bond angles of $\angle N_1 C_1 C_2$, $\angle N_5 C_5 C_6$ and $\angle C_1 C_5 C_6$, respectively, and the previous use of $\lambda_1$ and $\lambda_2$ are here replaced as $(\lambda_1)_{i+1} = -(\tau_i - \gamma + \pi)$ and $(\lambda_2)_{i+1} = \tau_{i+1}$. The above equation shows that $\tau_i$ and $\tau_{i+1}$ are only the variables when $\theta$ and $\gamma$ are fixed in advance. Therefore, all the $z$'s are successively determined when one of the peptide planes is fixed, although it may sometimes happen that no solution exists.

We have examined the effect of small disturbance at the $i$-th peptide plane ($\Delta \tau_i$) to see whether the distortion propagates equally in both ($N$-terminal and $C$-terminal) directions. Pauling-Corey geometry ($\xi=13.2^\circ$, $\eta=22.2^\circ$, $\alpha=111.5^\circ$) is employed for the peptide backbone, and the $C^\alpha$ atoms are fixed at those positions that the $\alpha$-helical structure has the lowest energy (i.e., $\varphi=-50.5^\circ$, $\varphi'=58.3^\circ$: $\theta=94.1^\circ$, $\gamma=46.3^\circ$: $\tau=-65.6^\circ$).

The distortion of $\alpha$-helical structure was also evaluated by energy calculation. Poly-L-alanine was chosen as the representative example for arbitrary side chains. Only the non-bonded interaction was taken into consideration (NH$\cdots$O hydrogen bond was simply set equal to zero except that the distance N$\cdots$O was smaller than 2.5 Å where the non-bonded repulsion was calculated). The total energy per residue for a sufficiently long regular structure is defined as the summation of pairwise interactions ($E_{ij}$) of the $i$-th residue with the other $j$-th residues ($i < j$)

$$E_i = \sum_{j>i} E_{ij} \quad (2)$$

The above expression was used as the energy of the $i$-th residue in a distorted $\alpha$-helix; the interactions with residues at the $N$-terminal side were neglected. This treatment will be rationalized later.

**Results of Calculations** Poly-L-alanine of 30 residues long was taken and a slight disturbance ($\Delta \tau = 1^\circ$ and $-1^\circ$) was imposed at the center of the helix (on the 15-th peptide plane). The change in the peptide rotational angle ($\Delta \tau$) and the energy difference ($\Delta E$) from those of the regular $\alpha$-helix were then calculated for each residue in both directions and plotted against the residue number in Fig. 2. It is clearly seen that the distortion of $\alpha$-helix increases rapidly toward the $N$-terminal while it converges to zero at the $C$-terminal side irrespective of the sign of the initial at the middle.

For the case of the negative disturbance (Fig. 2B), the solutions of Eq. 1 are obtained only for several residues in the $N$-terminal direction and no solution beyond the 12-th residue. This may arise from the severe restriction of the fixed positions of all the $C^\alpha$ atoms at the $\alpha$-helical conformation and the rigid geometry used for peptide backbone. However, the general tendency that the $N$-terminal side of the disturbed residue is more perturbed than the $C$-terminal side is obvious, and this feature may pertain to the real situation of $\alpha$-helix.
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Fig. 2. The change of the rotational angle, $\Delta \tau_i$ (---), and the energy difference, $\Delta E_i$ (---), of the $i$-th peptide unit from the α-helical conformation by the application of the distortion at the 15th residue. The initial distortion, $\Delta \tau_{15}$ was set $1^\circ$ (A) and $-1^\circ$ (B) respectively.

The energy difference of the $i$-th residue compared with that of the regular α-helix is also plotted in Fig. 2. The quantity, $\Delta E_i$, calculated with the use of Eq. 2 is interpreted as the excess energy at the N-terminal end of a distorted helical structure in which C-terminal portion always keeps the regular structure. Figure 3 is plots of the concomitant change of $\varphi_i$, $\psi_i$ with the distortion $\Delta \tau_i$. It shows the monotonous

Fig. 3. Concomitant change of $\varphi_i$ and $\psi_i$ with the distortion $\Delta \tau_i$ (initial distortion $\Delta \tau_{15}=1^\circ$).
movement along the contour line of $\theta =$ constant, while the directions are opposite depending on the sign of the initial disturbance of $A_r$. The path of the $\phi_1$, $\phi_2$ movement is almost in accordance with the trough of the low energy of poly-L-alanine, which may explain the fact that the energy change in Fig. 2 is not so large.

Asymmetric nature of polypeptide chain was also pointed out by Kotelchuck and Scheraga. They found from the analysis of a protein structure that a conformational state of one residue is mainly determined (in the sense of the short-range force only) by the interaction of its own side chain with the peptide group at the C-terminal side. It means that the orientation of a peptide group influences the neighboring residue at the N-terminal side but not one at the C-terminal side. This agrees with our finding described above. If the polarity of a polypeptide chain is the general case, we could expect some directionality in the $\alpha$-helical structure. The instability considered in the present model may be regarded as those amino acid residues so-called helix breaker. Since the N-terminal side of a given helix is much more disturbed, it should be the N-terminal portion rather than the C-terminal one, in which a helix-breaking residue would be incorporated more easily. This may explain the distribution of proline residues, which is counted as a strong helix breaker, in helical regions of 15 proteins; 18 prolines locate at either three sites of N-terminal region of the helix but none in the inner nor C-terminal helix region. The above line of deduction leads us to an expectation that the growth process of $\alpha$-helix proceeds strictly on the C-terminal direction (i.e., helix breaker cannot be incorporated but loosely in the N-terminal direction).

EXPERIMENTAL SECTION

Materials and Methods

Materials  Boc*-amino acids and polystyrene (2% divinylbenzene, 100–200 mesh) were purchased from Protein Research Foundation (Osaka, Japan). All amino acids except glycine were in l-form. $\gamma$-Benzyl-L-glutamate, purchased as a form of cyclohexyl-ammonium salt, was transformed into the free acid by a treatment with citric acid. Triethylamine was distilled over sodium metal before the use.

Chloromethylation  Polystyrene was chloromethylated by the method described by Merrifield. The amount of chloromethyl groups incorporated in the product resin was determined as follows. An aliquot (250 mg) of chloromethylated resin was suspended in 20 ml of pyridine, and heated for 3 hours at ca. 100°C. After being cooled, water (10 ml) and concentrated nitric acid (20 ml) were added to the suspension, and the amount of displaced chloride was determined by the Volhard titration. Two lots of chloromethylated resin were prepared and it was found that one lot (referred to resin A) contained 1.93 mmol of chloromethyl group per g of resin and the other lot (resin B), 0.70 mmol per g.

Preparation of Boc-glycyl-resin  4.73 g of Boc-glycine and 3.46 ml of triethylamine were added to 10 g of resin A in a mixture of 30 ml of ethanol and 15 ml of chloroform, and the mixture was stirred at room temperature for 1 hour and then heated at reflux

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* Abbreviations used: Boc-, t-Butyloxycarbonyl; TFA, trifluoroacetic acid; DCC, dicyclohexyl-carbodiimide; DNP-, dinitrophenylated; TLC, thin layer chromatography.
for 48 hours. The esterified resin was collected by filtration, and washed successively
with ethanol, acetic acid, water and methanol. The cycle was repeated several times.

An aliquot of Boc-glycyl resin A was hydrolyzed as described below. The amount
of amino acid released from the resin was estimated colorimetrically with ninhydrine,
and found to be 0.75 mmol per g of resin.

Boc-phenylalanine was reacted with Resin B in a similar way. We carried out
two runs: the first run gave a product called Phe-Resin B1, the second gave Phe-Resin
B2, and each was used for the trial of synthesis of (Ala)$_{20}$ (Glu)$_{20}$ Phe and (Glu)$_{20}$
(Ala)$_{20}$ Phe, respectively. The amount of phenylalanine bound to Resin B1 was
determined by a colorimetric assay with ninhydrine after acid hydrolysis and found to
be 0.39 mmol per g of resin. For Resin B2, acid hydrolysis and subsequent analysis
with an automatic amino acid analyzer (Jeol JLC3) under the condition of D. H.
Spackman et al.\textsuperscript{7)} gave a value of 0.31 mmol of phenylalanine per g of resin.

**Reaction cycle coupling of Boc-amino acids** 25 ml of solvent and 3 g of Boc-
glycyl-resin A or Boc-phenylalanyl-resin B1 (or B2) were placed in a reaction vessel for
solid-phase peptide synthesis. The inside diameter of the vessel was 3 cm, the height
7 cm, and the volume 50 cm$^3$. The vessel was fixed on a rod which swings right
and left 24 times per minute around a pivot. The swinging angle was 50° to one side
and 60° to the other side from the vertical line. A reaction cycle is shown in Table I.
All reactions were performed at room temperature.

<table>
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<tr>
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<th>Reagent</th>
<th>Time</th>
<th>Repeats</th>
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<td>2</td>
<td>Washing</td>
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<td>3</td>
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<td>3</td>
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<td>1</td>
</tr>
<tr>
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<tr>
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<td>2</td>
<td>3</td>
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<td>Deprotonating</td>
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<td>Washing</td>
<td>CH$_3$Cl$_2$</td>
<td>2</td>
<td>3</td>
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</table>

\textsuperscript{a)} equimolar reagent with the amount of C-terminal amino acid of polypeptide.

**Acid hydrolysis of polypeptide to resin** Resin with synthesized peptide in a mixture
of 0.5 ml of distilled HCl and 0.5 ml of propionic acid was hydrolyzed in an evacuated
and sealed ampoule at 110°C for 24–42 hours. When alanine content was high, 80%
aqueous TFA or TFA-conc. HCl (1:1) were used in parallel with 6N HCl-Propionic
acid. The highest value of liberated alanine was used to evaluate the content of
alanine.
**Quantitative Analysis of Amino Acid**

1. **Amino acid or peptide coupled to Resin A**  
An acid hydrolyzate, which was dried and freed from volatile acids, was dissolved in 3% aqueous sodium hydrogen carbonate-ethanol (1:2) containing 1% of 2, 4-dinitrofluorobenzene. The mixture was stood for 3 hours with occasional shaking. The products, dinitrophenylated amino acids, were analyzed by TLC. A solvent system, chloroform: methanol: acetic acid = 85:15:5, was used exclusively. A spot of DNP-amino acid was extracted with 4% sodium hydrogen carbonate. The amount of DNP-amino acid was calculated from the absorbance at 360 nm.

2. **Amino acid or peptide coupled to Resin B1 (or B2)**  
An acid hydrolyzate was analyzed by the amino acid analyzer. The ratios of glutamic acid and alanine to phenylalanine were calculated on an assumption that HW constant of each amino acid had the identical value. The assumption would hold if we concerned accuracy of ±5% for relative ratios of these amino acids.

In all cases no attempts were made to estimate a degree of racemization which might occur during the coupling reaction of Boc-amino acids.

**Quantitative analysis of free N-terminal of peptide resin**  
The amount of free N-terminal of peptide bound to resin, which was obtained after the step 8 of a reaction cycle (see Table I), was quantitatively determined by the methods described by Dorman or Esko, Karlsson, and Porath.  
As to the Porath’s method, solvent for the measurement of absorbance at 420 nm was modified: instead of original CH₂Cl₂ alone, a mixture of dimethylsulfoxide and CH₂Cl₂ (5:1) was used in order to avoid a change in concentration of chromophoric solute caused by the evaporation of CH₂Cl₂.

**Cleavage of peptide from peptide-resin**  
A mixture of Boc-(γ-benzyl-Glu)₁₁-Phe resin (223.5 mg), anisole (1 ml), and 25% hydrogen bromide in acetic acid (10 ml) was kept at room temperature for 2 hours with occasional shaking. Trifluoroacetic acid (10 ml) was added to this suspension and the mixture was stood for 1 hour. The residues obtained by filtration were washed with 10 ml of TFA. The filtrates were evaporated in vacuo to give a solid which was thoroughly washed with ether and dried over P₂O₅. The solid products were dissolved in 10 ml of 0.1 N sodium hydroxide and 0.5 ml of the solution was used for hydrolysis with 6 N HCl, 0.5 ml for dinitrophenylation, and 8 ml for further purification. The total hydrolyzate was analyzed with the amino acid analyzer.

**Dinitrophenylation of the free peptide**  
Dinitrophenylation was carried out with a standard procedure.  
TLC (chloroform: methanol: acetic acid = 95:5:1) of the products showed only DNP-glutamic acid as a DNP-amino acid. The number of free peptide was calculated from the yield of DNP-glutamic acid as 3.66×10⁻⁶ mol after correction necessary for a loss of DNP-glutamic acid during hydrolysis. The value corresponded to 1.6×10⁻⁶ mol of peptide per g of resin, and the amino group of the N-terminal glutamic acid could be deprotected with an acid treatment.

**Purification of the free peptide**  
A residue obtained by evaporation of 8 ml of the solution of the free peptide in vacuo was dissolved in 1 ml of water with the addition of a few drops of 1 N sodium hydroxide. The solution was fractionated on a column of Sephadex G-15 (1.0×150 cm), equilibrated and eluted with 0.1 N potassium chloride-0.05 M (in Na⁺) sodium phosphate buffer at pH 8.5. Fractions detected by the ninhydrin method after alkaline hydrolysis gave a chromatogram shown in Fig. 5.
The fractions 22-24 were pooled and desalted on a column of Sephadex G-10 with the elution by 0.1 M ammonium formate. The fractions containing peptides were combined and lyophilized. The product was dissolved in 0.05 M lutidine-HCl buffer at pH 6.70 and chromatographed on DEAE-cellulose (Whatman, type DE 32). Elution was performed with a linear gradient of sodium chloride concentration from 0.5 to 2.0 M in the same buffer and fractions of 2 ml were collected. Aliquots (0.3 ml each) were analyzed with ninhydrin after alkaline hydrolysis and the chromatogram was illustrated in Fig. 6. Fractions 85-90 were pooled and an aliquot was submitted to an amino acid analysis after hydrolysis with 6 N HCl.

Results of Synthesis

Confirmation of our calculation described before might be given by a study of conformational stabilities of block copolypeptides represented as $A_nB_n$ and $B_nA_n$, i.e., an inverted block sequence to one another. We took 20 as $n$ and selected glutamic acid and alanine for $A$ and $B$ according to the following reasons. First, both of the individual homopolymers composed of glutamic acid or of alanine can form $\alpha$-helices in an appropriate solution condition, and the number of the residues in these copolypeptides, 40, is expected to be long enough to form $\alpha$-helix. Second, the fact that glutamic acid has an acidic side chain and alanine has no ionizable one enables us to change the conformation of the glutamyl block in the copolymers by the pH of their solutions while the alanyl block is unaffected. The stability of an $\alpha$-helical structure in $(\text{Glu})_{20}(\text{Ala})_{20}$ and in $(\text{Ala})_{20}(\text{Glu})_{20}$ could then be compared to one another to see which polymer will change its conformation more easily when such a perturbing condition is applied.

The block copolypeptides, $(\text{Glu})_{20}(\text{Ala})_{20}$ and $(\text{Ala})_{20}(\text{Glu})_{20}$, might be synthesized possibly by a standard classical technique of peptide synthesis. We selected, however, the solid-phase method for that purpose because of the following reasons: (1) The weakest point ascribed to the method is that a coupling reaction is not always complete and therefore peptides lacking one or more residues are unavoidably coexisting in the products. In a standard liquid-phase method, where a purification procedure should be accompanied with each coupling steps, such undesired products might be excluded from the final product. Coexistence of peptides in which one or two residues are missing would not cause a serious trouble in our project, because conformational stability of those peptides would not be affected as much as expected in the case of physiological activities. It does not necessarily means that the homogeneity of a product is not a concern. Most efforts should be paid to improve the yield of homogeneous products. (2) There are only few reports on an application of the solid-phase method for synthesis of peptides having only one regular secondary structure. For the synthesis of those peptides the solid-phase method inevitably means a sequence of reactions at the end of linear molecules (after a regular secondary structure was attained) attached and embedded in solid matrice. The situation might be different for that experienced in synthesis of globular and nearly random-coiled peptides, and impose a problem important to the synthetic method itself. The method is still young and requested for a piling up of studies on synthesis of a variety of compounds in order to make the method valuable to be called as a “standard method”. Our attempt to synthesize $\alpha$-helical peptides would also contribute to a progress of the solid phase-
method in this sense. (3) The method is well-known for its simplicity in procedure. This is the most advantage employed here.

In the solid phase peptide synthesis all reactions are carried out in solid matrix and therefore the nature of a solid matrix is most significant. Beads of polystyrene-2% divinylbenzene as a solid support used and considered to be most appropriate in many studies. The reaction cycle (Table I) and reaction apparatus are also commonly employed one (see the reviews).

The solid-phase synthesis was begun with an introduction of Boc-glycine or Boc-phenylalalanine into polystyrene resin. Polystyrene was chloromethylated by the procedure described by Merrifield to give chloromethylated resin which was reacted with Boc-glycine or Boc-phenylalanine in the presence of triethylamine. The amount of glycine or phenylalanine incorporated in resin was 0.75–0.31 mmol per g of resin. These glycy1 or phenylalanyl residues served as a label of synthetic chains, we could calculate an amount of further incorporation of glutamyl or alanyl residues in a chain from a comparison with the amount of glycine (or phenylalanine) in a total acid hydrolyzate after each cycle of solid-phase synthesis had finished.

It is widely experienced that reactions in a heterogeneous system are poorly reproducible. This is the case for the present study, chloromethylation and an introduction of the first amino acid gave different products in each run. Two runs of chloromethylation of polystyrene gave two products which were called Resin A and Resin B and contained chloromethyl groups 1.93 mmol and 0.70 mmol per g of resin, respectively. Boc-glycine was introduced into Resin A, Boc-phenylalalanine into Resin B. Two experiments were carried out for the latter reaction; each run gave different products which were called Resin B1 and Resin B2, each contained 0.39 mmol and 0.31 mmol of phenylalanine per g of resin, respectively.

Resin A We tried to synthesize (γ-benzyl-Glu)20 (Ala)20Gly on Resin A. Aliquots, which were taken to determine the amount of amino acid incorporated after each reaction cycle of amino acid coupling had finished, were hydrolyzed with acid. The hydrolyzate was dinitrophenylated to give a mixture of DNP-amino acids which was analyzed quantitatively with TLC. The ratios of alanine and glutamic acid thus obtained were illustrated in Fig. 4. The first 20 cycles completed the alanyl block with good yield; 20.0 alanyl residues per chain were found. Three γ-benzyl-glutamyl residues could be further introduced by the 21st-23rd cycles to extend the peptide chain, then a sudden drop in yield of introduction of that residue at the 24th cycle and ever afterwards was observed.

Resin B1 Synthesis of (Ala)20(γ-benzyl-Glu)20Phe on Resin B1 was then tried. In this peptide the residues in the C-terminal half is composed of glutamic acid. The ratio of glutamic acid to phenylalanine, which was determined by the amino acid analyzer after total hydrolysis of an aliquot of the product obtained at the end of reaction cycles until 6th showing completeness of the reaction (see Fig. 4). After the 7th reaction cycle gradual decrease in slope of a Glu/Phe vs. cycle number plot was observed. The result seems to be incompatible with that of the previous experiments with resin A, but perhaps no remarks could be made because different resin was used and the reaction centers were located in different distance from a solid matrix in these two experiments.

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Fig. 4. The ratio of alanine or glutamic acid to C-terminal amino acid. 

\( \bullet - \bullet \), the synthesis on Resin A on which glycine was attached as the first amino acid; \( \bullet - \bullet \), the synthesis on Resin B1; \( \circ - \circ \), the synthesis on Resin B2. C-terminal amino acid of the second and the third polypeptide was phenylalanine. As to the first and the third, the ratio of alanine to glycine or phenylalanine was plotted up to the 20th cycle and that of glutamic acid after the 21st cycle. The ratio of glutamic acid to phenylalanine was plotted for the second one.

After the completion of the step 8 in the 11th cycle, the amount of the free N-terminals of the peptide was measured by Dorman's method and was found to be 54% of the value obtained at the same step in the first cycle. After completion of the 11th cycle a part of resin was taken and the peptide was cleaved from the resin with hydrogen bromide in acetic acid. One twentieth of the peptide was dinitrophenylated and another one twentieth was submitted to determination of amino acid composition. The yield of DNP-glutamic acid corresponding to the amount of the free N-terminal in the peptide, was found to be 57% of phenylalanine, and the value coincided approximately with the result of Dorman's method in the 11th cycle. The remaining peptide was fractionated on a column of Sephadex G-15 (Fig. 5) and aliquots of the pooled fraction 22–25, the fraction 28 and the fraction 34 were analyzed for their amino acid composition. The ratio of glutamic acid to phenylalanine was 11.6 in the fraction 22–24, 6.9 in the fraction 28, respectively. The fraction 34 contained too little amount of phenylalanine to calculate the amount. The pooled fraction 22–24 was desalted and further purified on a column of DEAE-cellulose (Fig. 6). Amino acid composition of the peptide in the pooled fraction 85–90 gave the ratio of glutamic acid to phenylalanine of 11.9.

Resin B2 This resin was used for the synthesis of the same peptide (except C-terminal amino acid) as with resin A to see the effect of different kind and population density of an anchoring amino acid on solid support. The ratio of alanine to phenylalanine at each cycle began to decrease from the expected value even at the 4th cycle, as seen
Fig. 5. The chromatogram of the polypeptides cleaved from Resin Bl. The solution containing cleaved peptides was laid on a column of Sephadex G-15 and eluted with 0.1 N-potassium chloride-0.05 M (in Na+)-sodium phosphate buffer at pH 8.5. The effluent fractions (2 ml) were analyzed by the ninhydrin method after alkaline hydrolysis and the absorbance at 570 nm was measured and plotted (— • —); magnified 10 times in absorbance scale (— O —).

Fig. 6. The purification of the fractions 22–24 in the chromatogram in Fig. 5 on DEAE-cellulose. The peptide was eluted with 0.05 M lutidine-hydrochloric acid buffer at pH 6.70 having a linear gradient of sodium chloride concentration from 0.5 to 2.0 M. Aliquots of the fractions were analyzed with the same method as described in the legend of Fig. 5.
in Fig. 4. However, even after the 24th cycle, Boc-γ-benzyl-glutamic acid could successively couple to extend the peptide chain, although with more reduced yield. Nevertheless the final yield of the desired tetracontapeptide was readily expected to be too low to continue the synthesis.

Possible reasons why the block copolypeptides could not be synthesized These results forced us to abandon the synthesis of the copolypeptides with the present method. The reasons of the failure has been considered as follows.

1. Possibility of incomplete cleavage of Boc group Several arguments on this point have been available and also considered in this study. For example, hydrogen chloride saturated in dimethylsulfoxide, which was reported to be most effective in the study of Chou et al.,15) was used instead of 50% TFA in dichloromethane at the step 3 in the 12th coupling cycle carried out on Resin B1. The amount of free N-terminal obtained by this treatment was still less than 50% of that of phenylalanine. Prolonged treatment up to 1 hour with 50% TFA in dichloromethane gave no improvements. It does not necessarily means, however, that the cleavage of Boc group proceeded completely and possibility of incomplete reaction by any means still remained.

2. Possibility of incomplete DCC reaction would be most probable. DCC reaction has been usually considered very rapid, and actually Anfinsen et al. gave an evidence that only one or two minutes were required to complete the reaction in their study in the synthesis of bradykinin.16) The reaction is, however, sluggish if a bulky amino acid such as leucine was concerned. For instance, DCC coupling of bulky amino acids to leucyl residues directly attached on a solid support was very slow.17) DCC reaction is composed of a nucleophilic attack of a free N-terminal amino group of peptide resin to a DCC-activated acyl component. In the present case we had to consider the additional fact that the nucleophilic group was located at the end of a peptide chain which would have fairly rigid α-helical conformation when a chain had ten or more residues. The combined effect of a fixed conformation around the reaction center and a bulky protecting group on the γ-carboxyl group of glutamic acid was considered to be most responsible for the present failure of the synthesis.

3. Pyroglutamyl residue formation was also possible to decrease the number of the nucleophilic N-terminal. Diborane will reduce pyroglutamyl group, if present, to prolyl residue which is readily checked for its presence.18) Absence of pyroglutamyl residue in the peptide in chromatographic reaction 85–90 on DEAE-cellulose was demonstrated since no detectable amount of proline was obtained by the treatment with diborane. Although further checks on the other fractions were performed, the fact that the number of free N-terminals was only 57% of that of crude peptide chains suggested the presence of N-blocked peptides by formation of pyroglutamyl or another means in some of them.

4. Properties of solid support will play a significant role in the solid phase synthesis. Although 2% crosslinked resin was used in the present study, 1% crosslinked resin was hopeful and should have been tested. Since reactions are carried out in solid matrices, coarse matrix will be more favorable for approach of reagents to a reaction center.
CONCLUDING REMARKS

Calculations revealed a unidirectional character of stability of α-helix. The failure in the synthesis of block copolypeptides still left a problem to confirm the result of theoretical calculations on experimental basis.

Recently, Bonora et al. reported their failure to synthesize (Glu)$_n$ by the solid-phase method if $n$ was greater than 4. Although their results are not comparable with ours as different resin and anchoring amino acid were used, difficulty of the synthesis have been well demonstrated by these studies.

It is concluded that the synthesis of block copolypeptides projected in the present study must be achieved by another approach. Preliminary study showed that the desired peptides could be obtained if Boc-oligopeptides instead of Boc-amino acids were used in the solid-phase method. We are still hoping to solve the problem in the near future.

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