Synthesis of Sequential Polypeptides of L-Leucine and Glycine

Takayoshi Iio and Sho Takahashi*

Department of Physics, Faculty of Science, Nagoya University, Nagoya 464

**Institute for Chemical Research, Kyoto University, Uji, Kyoto 611*

(Received May 7, 1974)

The following sequential polypeptides of L-leucine and glycine were synthesized: $(Leu_3Gly)_n$, $(Leu_2Gly)_n$, $(LeuGly)_n$, and $(LeuGly_2)_n$. Polymerization was achieved by self-condensation of the corresponding peptide *p*-nitrophenyl esters. Hydrolysis of the polypeptides were carried out in 90% aqueous trifluoroacetic acid and the time course of liberation of leucine from each polymer was presented. Apparent first-order rate constants ($\times 10^2$), 1.93, 2.86, 2.91, and 4.4 hr^{-1} were obtained for liberation of leucine from $(Leu_3Gly)_n$, $(Leu_2Gly)_n$, $(LeuGly)_n$, and $(LeuGly_2)_n$, respectively.

A number of studies have been made with homo- or random copolypeptides as a model system to understand the secondary structure of protein.[1] Many basic conclusions derived in those works are useful to investigate protein structure including a prediction,[2] their application, however, is surely limited to only qualitative ground. Interactions between side chains of amino acids in proteins are so much complicated that the studies of sequential polypeptides, in which side chains of amino acids are arranged periodically to amplify interactions between them, are desired and will be promising to analyze the interaction quantitatively. With this aspect we previously reported the studies on $(L\text{-}Ala_xGly_y)_n$,[3] it was considered to be necessary to extend the work with sequential polypeptides having much larger side chain. In this paper we will describe the synthesis of $(L\text{-}Leu_xGly_y)_n$ and the behaviors of some leucine polypeptides toward acid hydrolysis, the succeeding paper will deal the conformational studies of the polypeptides in solution.[4]

Results and Discussion

Synthesis. The synthesis of sequential polypeptides having a defined repeating unit of amino acids are usually achieved by a self-condensation of unit peptide active esters, the method has been considered the best general one.[5] Other methods, such as a treatment of peptide free acids with tetraethyl- or bis-*o*-phenylenepyrophosphite,[6] usually required more drastic condition. Among the *p*-nitrophenyl-,[5] pentachlorophenyl-,[7] and *N*-hydroxysuccinimidyl-esters,[8] which are the most widely used active esters, we preferred the *p*-nitrophenyl esters on account of their good crystallinity over *N*-hydroxysuccinimidyl esters. Lorenzi *et al.*[9] compared the polymerization of *N*-hydroxysuccinimidyl- and *p*-nitrophenyl-esters of peptide in the course of their synthesis of $(AlaProGly)_n$, and showed that there were no differences in both products. Our previous experiences on a number of phenyl esters[10] and peptide esters[3] showing *p*-nitrophenyl esters more soluble than pentachlorophenyl esters supported the use of the former: high solubility of the peptide active esters was essential to avoid intramolecular cyclization during the polymerization.

Synthetic steps to the required polymers are shown in Fig. 1. An attempted synthesis of the tripeptide ester (XVIII) *via* azide coupling of (I) and the hydra-

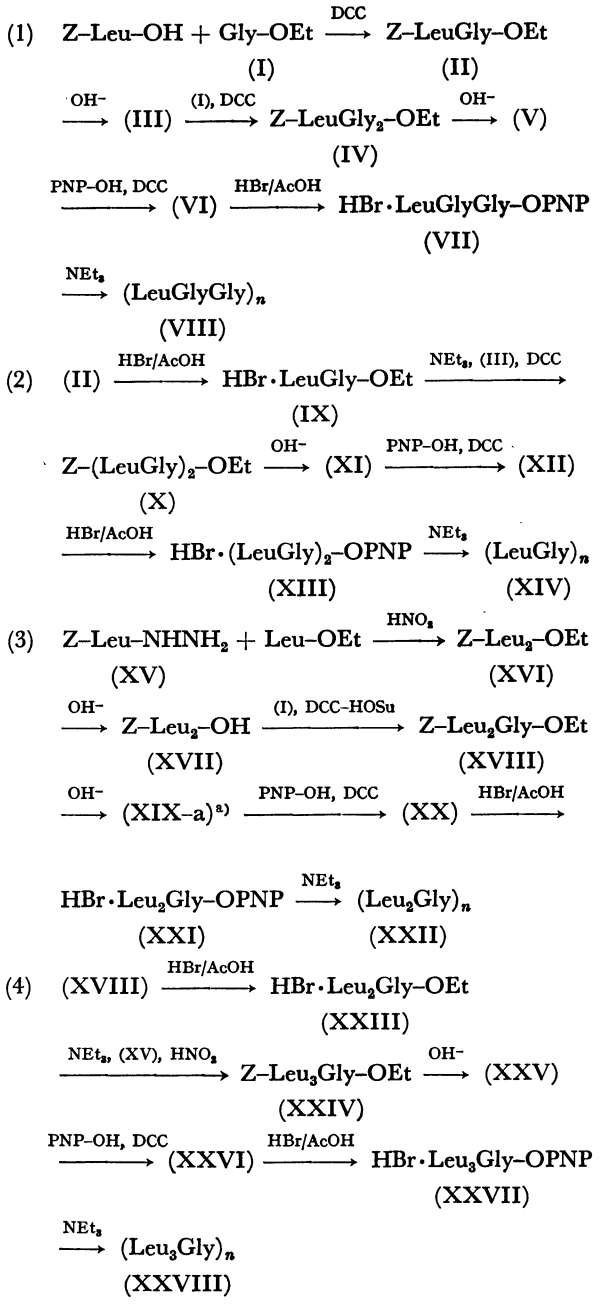

Fig. 1. Steps to the polypeptides, $(Leu_xGly_y)_n$. Abbreviations: Z=benzyloxycarbonyl, PNP=*p*-nitrophenyl. a) Characterized as its dicyclohexylammonium salt, (XIX).

zide of (XVII) in hope to minimize a possibility of racemization of terminal leucine was failed in preparative aspects since the azide derived from XVII was poorly soluble. The use of dicyclohexylcarbodiimide (DCC) in the presence of an equimolar amount of *N*-hydroxysuccinimide (HOSu) was then satisfactorily introduced for a preparative purpose, the resulting XVIII was not distinguished from the product of the azide method in respect of mp; without HOSu, the product had a mp about 10 degrees lower and resisted to further purification.

Concentrated solutions (30—40% in dimethyl sulfoxide) of the polymerizing unit peptide active ester hydrobromides (VII, XIII, XXI, XXVII) were neutralized with triethylamine to afford the polypeptides (VIII, XIV, XXII, XXVIII). The weight average molecular weight was roughly estimated as 30000, 20000, 25000, and 20000 for VIII, XIV, XXII, and XXVIII, respectively, from intrinsic viscosity in dichloroacetic acid.[11]

Hydrolysis of Polypeptides. Estimation of chemical and optical purities of synthetic polypeptides is exclusively achieved by an amino acid analysis and by a measurement of optical rotation on hydrolyzates. The most general condition, hydrolysis in 6 M-hydrochloric acid,[12] was not applicable to the present case by the fact that poor solubilities of leucine polypeptides whose leucine contents were high as in XXII and XXVIII in 6 M or concd hydrochloric acid caused the rate of hydrolysis very slow (after 5 days hydrolysis at 110 °C still precipitates were observed). Among literatures of the synthesis of poly-leucine only two papers reported total hydrolysis: one in 6 M- (40 hr)[13] and the other in 12 M-hydrochloric acid (3—4 days)[14] without comment of results. Other substances used for

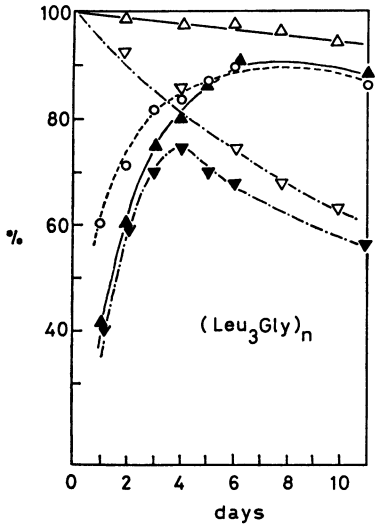

Fig. 2. Hydrolysis of $(Leu_3Gly)_n$ in 90% aq. TFA at 110 °C. Values are given as % of the theoretical values. ○------○: liberation of glycine from the polymer, ▲——▲: liberation of leucine from the polymer, ▼·····▼: optical rotation (at 300 nm) of isolated leucine, △——△: amount of recovered leucine when pure L-leucine was treated in the same condition, and ▽·····▽: optical rotation of L-leucine treated in the same condition.

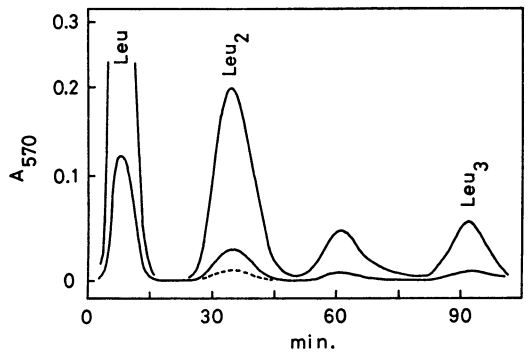

Fig. 3. Amino acid analysis of the hydrolysates of $(Leu_3Gly)_n$. Hydrolysis was carried out in 90% aq. TFA for 3 days (solid line) and 6 days (dotted line). The analyzer column was operated at the standard condition until just berore leucine was eluted, then pH 5.28 buffer was delivered (noted as the time zero on the abscissa). The lower curve was magnified 10 times to yield the upper one.

total hydrolysis, mono-,[15] di-,[16] tri-chloroacetic acid,[15] formic acid,[15] and oxalic acid,[15,17] did not give better results. After looking for another hydrolytic media, especially among perfluoro-acetic, -propionic, and -butyric acid, 90% aqueous trifluoroacetic acid (TFA) was finally adopted as a medium of hydrolysis, and further experiments were carried out with this solvent. The other 90% aqueous perfluoroaliphatic acids or 1 : 1 mixtures of fluoroacids and concd hydrochloric acid gave approximately similar results in the respect of amino acids liberation upon hydrolysis. Figure 2 illustrated the typical results of the hydrolysis of XXVIII, where liberation of glycine and leucine, values of molecular rotation (at 300 nm) of leucine which was separated on a chromatographic column from the hydrolyzates, and the corresponding results obtained in control experiments were shown. The well-known facts that the rate of hydrolysis of a peptide bond Leu–Leu was about a tenth to a hundredth of those of Leu–Gly, Gly–Leu,[18] and Gly–Gly,[18,19] were clearly reflected in an amino acid analysis of the hydrolyzates (Fig. 3). Figure 3 showed the appreciable amount of Leu_2 and Leu_3 was present in the hydrolyzates. Such fragments also might contribute the molecular rotation in an appreciable manner, this is the reason why optical rotation was measured with isolated leucine itself. Although the molecular rotation of leucine decreased fairly rapidly in this hydrolytic condition (half life 14 days), extrapolation to zero hydrolysis time established that the racemization in XXVIII should be less than 5%. The other polypeptides, VIII, XIV, and XXII, also gave the same result.

Each leucyl peptide bond in the unit (Leu_xGly_y) might have a different rate constant for hydrolysis and thus the appearance of free leucine in the hydrolyzates should be described by a composite of these rate constants.[20] But the actual liberation of leucine from VIII, XIV, XXII, and XXVIII was found to be approximately represented by apparently single first-order kinetics as shown in Fig. 4, at least short period

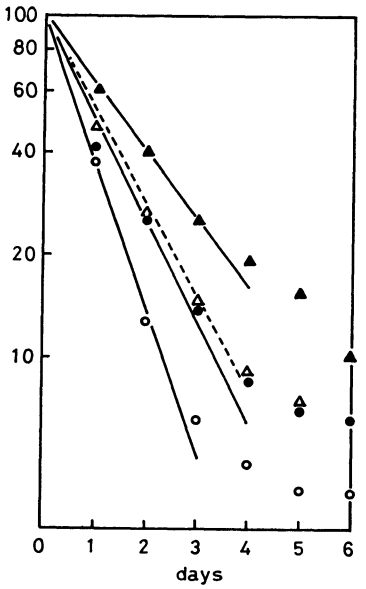

Fig. 4. Semilogarithmic plot of hydrolysis data. The values of 100-x (x is the amount of free leucine yielded from hydrolysis) are plotted against hydrolysis time. ▲——▲: $(Leu_3Gly)_n$, △------△: $(Leu_2Gly)_n$, ●——●: $(LeuGly)_n$, and ○——○: $(LeuGly_2)_n$.

of hydrolysis time was concerned. The estimated apparent-first order rate constants ($\times 10^2$) for appearance of free leucine are 1.93, 2.86, 2.91, and 4.4 hr^{-1} for VIII, XIV, XXII, and XXVIII, respectively, those values would serve as a measure of hydrolysis of leucyl residues from polypeptides.

Experimental

TFA, DCC, and HOSu were purchased from Protein Research Foundation, Osaka. L-Leucine and other reagents were of the purest or reagent grade of commercially available products. All melting points are uncorrected values on a micro hot plate. Viscosities were measured at 25 °C using an Ostwald type viscometer. Optical rotation was measured at 300 nm with a JASCO ORD/UV-5.

Coupling with DCC. DCC reactions were usually carried out at −8 to 0 °C for 4—5 hr, then at 5—8 °C for 12—48 hr with stirring. 1.1 molar equivalent of DCC and 1.2 to 1.5 equivalent of *p*-nitrophenol or an amine component, which was obtained by treating amino acid or peptide ester salts with 1.05 equivalent of NEt_3, were used.[22]

Reaction with HBr/AcOH. Ten mmol of a peptide *p*-nitrophenyl ester was treated with 4 ml of 25% HBr in acetic acid at room temperature for 1—4 hr.

Saponification. NaOH (1.2 equimolar amount) was used.

Hydrolysis. Polypeptide (5 to 10 mg) was suspended in 3 ml of 90% aq. TFA in an ampoule, cooled with liquid nitrogen to solidify, evacuated to 10^{-2} Torr, sealed, and kept at 110 °C. Hydrolyzates were evaporated to dryness *in vacuo*, the residue was dissolved in 0.001 M HCl and analyzed on a JEOL liquid chromatography system according to the method of Spackman *et al.*[21] A JEOL separation system JLC-3 was used to isolate free leucine in hydrolyzates.

Z-Leu-Gly-Gly-OEt (IV). The compound was prepared from (III)[23] and (I) by a DCC reaction in $CHCl_3$ and recrystallized from ethyl acetate–cyclohexane. Yield, 87%, mp 103—105 °C.

Found: C, 59.25; H, 7.25; N, 10.51%. Calcd for $C_{20}H_{29}O_6N_3$: C, 58.95; H, 7.17; N, 10.31%.

Z-Leu-Gly-Gly-OPNP (VI). IV was saponified in methanol with 1.2 M NaOH to afford V, which was esterified by a DCC reaction in tetrahydrofuran. VI was recrystallized from ethyl acetate–ether. Yield, 53%, mp 136—138 °C.

Found: C, 57.73; H, 5.71; N, 11.79%. Calcd for $C_{24}H_{28}O_8N_4$: C, 57.59; H, 5.64; N, 11.20%.

HBr·Leu-Gly-Gly-OPNP (VII). VI was treated with HBr/AcOH to afford VII, which was recrystallized from ethanol–ether. Yield, 48%, mp 191—195 °C (dec.).

Found: C, 43.17; H, 5.19; N, 12.83%. Calcd for $C_{16}H_{23}O_6N_4Br$: C, 42.96; H, 5.18; N, 12.53%.

Z-Leu-Gly-Leu-Gly-OEt (X). II[23] was treated with HBr/AcOH to give IX, which was coupled with III by a DCC reaction in $CHCl_3$. Yield, 85%, mp 157—159 °C (ethanol–ether).

Found: C, 59.72; H, 7.83; N, 11.03%. Calcd for $C_{26}H_{40}O_7N_4$: C, 59.98; H, 7.74; N, 10.76%.

Z-Leu-Gly-Leu-Gly-OPNP (XII). The compound X was saponified in dioxane with 0.43 M NaOH to give XI (obtained as amorphous powder), which was coupled with *p*-nitrophenol by a DCC reaction in tetrahydrofuran. Yield. 67%, mp 168—173 °C (ethanol–ether).

Found: C, 59.80; H, 6.64; N, 12.27%. Calcd for $C_{30}H_{39}O_9N_5$: C, 58.72; H, 6.43; N, 11.41%.

HBr·Leu-Gly-Leu-Gly-OPNP (XIII). XIII, obtained from XII with HBr/AcOH, was recrystallized from ethanol or dioxane. The compound was highly hygroscopic. Yield, 85%, mp 125 °C.

Found: C, 47.05; H, 6.25; N, 12.27%. Calcd for $C_{22}H_{34}O_7N_5Br$: C, 47.15; H, 6.11; N, 12.50%.

Z-Leu-Leu-OEt (XVI). 20 g of Z-Leu-hydrazide (XV) in 150 ml of 1 M HCl and 60 ml of acetic acid was treated with 5.3 g of $NaNO_2$ at −5 °C. The azide was extracted with ether and reacted at the same temperature for 2 hr with ethyl leucinate which was prepared from 19 g of the hydrochloride and 9.9 g of NEt_3. The reaction mixture was kept at 0 °C for 24 hr, then worked up as usual. Yield, 21 g, mp 90—91 °C (ethyl acetate–cyclohexane) (lit, 89—90 °C[25]).

Z-Leu-Leu-Gly-OEt (XVIII). XVI was saponified in acetone with 1 M NaOH to give (XVII) (mp 102—103 °C, lit, 98—101 °C[26]). To a mixture of 14.8 g of I hydrochloride, 10.7 g of NEt_3, and 200 ml of tetrahydrofuran, 36.3 g of XVII and 12.1 g of HOSu were added. The whole mixture was cooled to −15 °C, 22 g of DCC in 70 ml of tetrahydrofuran was added portionwise. After standing at −15 °C for additional 5 hr, then at 0 °C for 2 day, the reaction mixture was worked up as usual. The compound was also obtained from XV and IX or from Z-Leu-Leu-hydrazide[24] and I by the method described in the preparation of XVI. In the last case, treatment of the hydrazide with $NaNO_2$ in HCl–AcOH instantaneously separated the Z-Leu-Leu-azide, part of which was extracted with $CHCl_3$ and was reacted with I. Yield, 29 g, mp 127—128 °C (lit, 111—112 °C[27]) (Found: C, 62.25; H, 8.11; N, 9.26%).

Z-Leu-Leu-Gly-OH, Dicyclohexylammonium Salt (XIX). XVIII was treated in acetone with 1.2 M NaOH to afford the free acid, which was converted into dicyclohexylammonium salt. Yield, 67%, mp 131—133 °C (ethanol).

Found: C, 66.64; H, 9.33; N, 9.47%. Calcd for $C_{34}H_{56}O_6N_4$: C, 66.20; H, 9.15; N, 9.08%.

Z-Leu-Leu-Gly-OPNP (XX). The compound was prepared from XIX and *p*-nitrophenol by a DCC reaction in tetrahydrofuran. Yield, 75%, mp 125 °C (ethanol or ethyl acetate).

Found: C, 60.57; H, 6.60; N, 10.05%. Calcd for $C_{28}H_{36}O_8N_4$: C, 60.42; H, 6.52; N, 10.07%.

HBr·Leu-Leu-Gly-OPNP (XXI). XX was treated with HBr/AcOH to afford XXI, which was recrystallized from ethanol. Yield, 74%, mp 234 °C (dec.).

Found: C, 47.60; H, 6.18; N, 11.10%. Calcd for $C_{20}H_{31}O_6N_4Br$: C, 47.72; H, 6.21; N, 11.13%.

HBr·Leu-Leu-Gly-OEt (XXIII). The compound was prepared from XVIII and HBr/AcOH. Yield, 93%, mp 166—167 °C (ethanol–ether). Found: C, 46.60; H, 7.61; N, 10.08%. Calcd for $C_{16}H_{32}O_4N_3Br$: C, 46.84; H, 7.86; N, 10.24%.

Z-Leu-Leu-Leu-Gly-OEt (XXIV). XXIII (22.4 g) in 100 ml $CHCl_3$ was treated at 0 °C with 5.56 g of NEt_3. The filtrate was added at 0 °C to Z-Leu-azide in 200 ml of ether prepared from 17 g of XV, the solution was kept at 0 °C for 2 day and worked up as usual. Yield, 66%, mp 235—236 °C (ethanol–tetrahydrofuran).

Found: C, 62.36; H, 8.55; N, 9.49%. Calcd for $C_{30}H_{48}O_7N_4$: C, 62.49; H, 8.39; N, 9.72%.

Z-Leu-Leu-Leu-Gly-OH (XXV). XXIV was treated in dioxane with 1 M NaOH to give XXV. Yield, 47%, mp 118—120 °C (tetrahydrofuran–cyclohexane).

Found: C, 61.65; H, 8.00; N, 10.46%. Calcd for $C_{28}H_{44}O_7N_4$: C, 61.27; H, 8.09; N, 10.21%.

Z-Leu-Leu-Leu-Gly-OPNP (XXVI). XXV was coupled with *p*-nitrophenol by a DCC reaction in dimethylformamide–dioxane (1:3). Yield, 50%, mp 208 °C (ethanol).

Found: C, 61.13; H, 7.26; N, 10.51%. Calcd for $C_{34}H_{47}O_9N_5$: C, 60.97; H, 7.07; N, 10.46%.

HBr·Leu-Leu-Leu-Gly-OPNP (XXVII). Prepared from XXVI and HBr/AcOH and recrystallized from dioxane–ether. Yield, 61%, mp 187 °C (dec.).

Found: C, 50.24; H, 6.89; N, 11.08%. Calcd for $C_{26}H_{42}O_7N_5Br$: C, 50.65; H, 6.87; N, 11.36%.

Polymerization of VII, XIII, XXI, and XXVII. 1.1 molar equivalent of NEt_3 was added to a 30—40% solution of VII, XIII, XXI, and XXVII in dimethyl sulfoxide under vigorous stirring. The mixture solidified within several minutes. After standing at room temperature for 2 days, the products were washed with a sufficient volume of ether, dissolved in TFA, and precipitated by adding ether. The procedure of dissolving and precipitation was cycled four times, the precipitates were dried over P_2O_5 and KOH. Yield, 40—60%.

Poly-(Leu-Gly-Gly) (VIII). Viscosity in dichloroacetic acid (DCA): $\eta_{sp}/c=0.267$ ($c=1\%$), $\eta_{int}=0.262$. Amino acid analysis: Leu: Gly=1.0: 1.99 (after 3 day hydrolysis).

Found: C, 52.34; H, 7.61; N, 18.67%. Calcd for $C_{10}H_{17}O_3N_3$: C, 52.85; H, 7.54; N, 18.49%.

Poly-(Leu-Gly) (XIV). Viscosity in DCA: $\eta_{sp}/c=0.151$ ($c=1\%$), $\eta_{int}=0.150$. Amino acid analysis: Leu: Gly=1.0: 0.99 (3 day hydrolysis).

Found: C, 54.13; H, 8.02; N, 15.88%. Calcd for $C_8H_{14}O_2N_2$: C, 56.45; H, 8.29; N, 16.46%.

Poly-(Leu-Leu-Gly) (XXII). Viscosity in DCA: $\eta_{sp}/c=0.221$ ($c=1\%$), $\eta_{int}=0.198$. Amino acid analysis: Leu: Gly=2.0: 0.99 (3 day hydrolysis).

Found: C, 58.89; H, 8.79; N, 15.40%. Calcd for $C_{14}H_{25}O_3N_3$: C, 59.34; H, 8.89; N, 14.83%.

Poly-(Leu-Leu-Leu-Gly) (XXVIII). Viscosity in DCA: $\eta_{sp}/c=0.159$ ($c=1\%$), $\eta_{int}=0.158$. Amino acid analysis: Leu: Gly=3.0: 0.94 (6 day hydrolysis).

Found: C, 58.97; H, 9.00; N, 13.92%. Calcd for $C_{20}H_{36}O_4N_4$: C, 60.58; H, 9.15; N, 14.13%.

We are greatly indebted to Dr. N. Kato at Nagoya University for his kind arrangement of elementary analysis. Thanks are also due to Prof. K. Yamasaki at the same university who kindly made available an ORD spectrometer for the experiment.

References

1) N. Lotan, A. Berger, and E. Katchalski, *Ann. Rev. Biochem.*, **41**, 869 (1972).
2) P. Y. Chou and G. D. Fasman, *Biochemistry*, **13**, 211 (1974).
3) S. Takahashi, This Bulletin, **42**, 521 (1969); T. Iio and S. Takahashi, *ibid.*, **43**, 515 (1970); T. Iio, *Biopolymers*, **10**, 1583 (1971); T. Iio and S. Takahashi, *Bull. Inst. Chem. Res. Kyoto University*, **49**, 80 (1971).
4) T. Iio and S. Takahashi, This Bulletin, in press.
5) D. F. DeTar, W. Honsberg, U. Honsberg, A. Wieland, M. Gouge, H. Bach, A. Tahara, W. S. Briniger, and F. F. Rogers, Jr., *J. Amer. Chem. Soc.*, **85**, 2873 (1963); F. H. C. Stewart, *Aust. J. Chem.*, **18**, 887 (1965).
6) H. Kitaoka, S. Sakakibara, and H. Tani, This Bulletin, **31**, 802 (1958); E. Heidemann and H. W. Bernhardt, *Nature*, **216**, 263 (1967); M. L. Huggins, K. Ohtsuka, and S. Morimoto, *J. Polym. Sci., Part C*, **23**, 343 (1968).
7) J. Kovacs, R. Giannotti, and A. Kapoor, *J. Amer. Chem. Soc.*, **88**, 2282 (1966).
8) B. B. Doyle, W. Traub, G. P. Lorenzi, F. R. Brown, III, and E. R. Blout, *J. Mol. Biol.*, **51**, 47 (1970); G. Ramachandran, A. Berger, and E. Katchalski, *Biopolymers*, **10**, 1829 (1971); R. Katakai, F. Toda, K. Uno, Y. Iwakura, and M. Oya, *Chem. Lett.*, **1973**, 763.
9) G. P. Lorenzi, B. B. Doyle, and E. R. Blout, *Biochemistry*, **10**, 3046 (1971).
10) L. A. Cohen and S. Takahashi, *J. Amer. Chem. Soc.*, **95**, 443 (1973).
11) P. Doty, J. H. Bradbury, and A. M. Holtzer, *ibid.*, **78**, 947 (1956); J. C. Mitchell, A. E. Woodward, and P. Doty, *ibid.*, **79**, 3955 (1957).
12) S. Moore and W. H. Stein, *Meth. Enzymol.*, **6**, 819 (1963).
13) K. D. Kopple and J. J. Katz, *J. Amer. Chem. Soc.*, **78**, 6199 (1956).
14) H. E. Auer and P. Doty, *Biochemistry*, **5**, 1708 (1966).
15) N. Maravalhas, *J. Chromatogr.*, **50**, 413 (1970).
16) R. E. Whitfield, *Science*, **142**, 577 (1963).
17) F. Feigl, *Angew. Chem.*, **70**, 1966 (1958).
18) R. L. Synge, *Biochem. J.*, **39**, 351 (1945); R. Hirohata, Y. Kanda, M. Nakayama, N. Izumiya, A. Nagamatsu, J. Ono, S. Fujii, and M. Kimitsuki, *Z. Physiol. Chem.*, **295**, 368 (1953).
19) K. Heyns, W. Walter, and H. F. Grützmacher, *J. Polym. Sci.*, **30**, 573 (1958).
20) D. A. Long and T. G. Truscott, *Trans. Faraday Soc.*, **59**, 2316 (1963).
21) D. H. Spackman, W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
22) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. II, John Wiley & Sons, New York, *N. Y.* (1961), p. 763.
23) M. Bergmann, L. Zervas, and J. S. Fruton, *J. Biol. Chem.*, **111**, 225 (1935).
24) CIBA Ltd., Ger. 1112525 (1962).
25) H. Aoyagi, M. Kondo, and N. Izumiya, This Bulletin, **41**, 2772 (1968).
26) E. L. Smith, D. H. Spackman, and W. J. Polgrase, *J. Biol. Chem.*, **199**, 801 (1952).
27) P. M. Hardy, G. W. Kenner, and R. C. Sheppard, *Tetrahedron*, **19**, 95 (1963).