*Synthesis of Poly-(L-prolyl-L-leucyl-glycyl). An Attempted Synthesis of Model Collagen**

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The presence of proline residues in a polypeptide chain has two important influences upon its chain configuration. Firstly, the lack of hydrogen atom on the pyrrolidine imide nitrogen makes it impossible to form the hydrogen linkage in the molecule. Secondly, the presence of pyrrolidine rings in the polypeptide chain reduces the degree of freedom of its internal rotation. These effects are supposed to be primarily responsible for the special chain configuration of collagen, gelatin and proline-rich fibrous proteins.

The structural investigations of collagen have been continued by many workers. On the basis of the amino acid analysis[1], it seems to be reasonable that collagen may consist of the polypeptide containing statistically a repeating unit of (glycyl-L-prolyl (or hydroxyprolyl)-X), where X represents any one of amino acid residues other than glycine and proline (or hydroxyproline). Furthermore, the presence of scattering units corresponding to the three amino acid residues in natural collagen was confirmed by X-ray analysis[2]. Following the above considerations, it seems most reasonable to regard the polypeptide with a regular repeating unit of (glycyl-L-prolyl-X) as a simplified model of collagen. Berger et al.[3], prepared poly-L-proline by the *N*-carboxy anhydride method, and the chain configuration of the polypeptide was determined by Cowan and McGavin[4] through X-ray analysis. This polyproline, however, is far from the model collagen.

In principle, the synthesis of high molecular weight polypeptide with a regular repeating unit of two or more amino acid residues is not impossible by the polymerization of tri- or higher membered peptide. For example, tri- or tetrapeptide ester[5], azide[6], thioester[7], mixed anhy-

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1) J. H. Bowes and R. H. Kenten, *Biochem. J.*, **43**, 358 (1948); W. A. Schroeder et al,. *J. Am. Chem. Soc.*, **76**, 3556 (1954).

2) M. L. Huggins, ibid., **76**, 4045 (1954).

3) A. Berger, J. Kurtz and E. Katchalski, ibid., **76**, 5552 (1954).

4) P. M. Cowan and S. McGavin, *Nature*, **176**, 501 (1955).

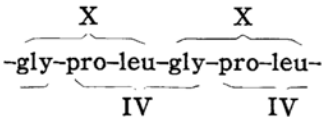
5) E. Pacsu and E. J. Wilson, Jr., *J. Org. Chem.*, **7**, 117, 126 (1942).

6) M. Z. Magee and K. Hofmann, *J. Am. Chem. Soc.*, **71**, 1515 (1949); M. Winitz and J. S. Fruton, ibid., **75**, 3041 (1953).

7) T. Wieland and H. Bernhard, *Ann.*, **582**, 218 (1953).

dride[8], carbothiophenyl derivative[9] and so on are polymerizable into polypeptides and tri- or higher-membered free peptides are polymerizable by the action of some peptide linkage forming reagents such as tetraethyl pyrophosphite[10] and dicyclohexyl carbodiimide[11]. However, these methods contain the hazard of forming ring peptides besides the main product of polypeptides. Then, the optimum condition for high molecular weight polymerization must be investigated in each case where the starting material may be different in solubility and reactivity.

In this paper, the synthesis of poly-(L-prolyl-L-leucyl-glycyl) was attempted in several ways. Firstly, the tripeptide, L-prolyl-L-leucylglycine(IV), and its derivatives were prepared, and the relative efficiencies of polycondensation methods involving dicyclohexyl carbodiimide, tetraethyl pyrophosphite and *p*-nitrothiophenylester methods were compared. Secondly, the relative reactivity for polycondensation was compared between L-prolyl-L-leucylglycine (IV) and glycyl-L-prolyl-L-leucine (X). Although two tripeptides, IV and X, differ in the position of proline residue in the molecule, it must be expected that they should give polypeptides with the same amino acid sequence with the only exception of both *N*- and *C*-terminal amino acid residues.



Synthesis of tripeptides and their derivatives. — Carbobenzoxy-L-prolyl-L-leucylglycine (III) was prepared according to the description of Ressler and du Vigneaud[12], and the protecting group was removed by hydrogenation in the presence of palladium-charcoal.

The tripeptide thioester, L-prolyl-L-leucylglycine *p*-nitrophenylthioester hydrobromide, was prepared by the procedure of Schwyzer[13]. Such a peptide *p*-nitrophenyl thioester was used for the preparation of the cyclic peptide by kenner[14].

The synthesis of ethyl carbobenzoxyglycyl-L-prolyl-L-leucinate (VIII) was accomplished by coupling carbobenzoxyglycyl-L-proline with ethyl L-leucinate through both mixed anhydride[15] and carbodiimide procedures[16]. After saponification of the product, the carbobenzoxy tripeptide (IX) was hydrogenated into glycyl-L-prolyl-L-leucine (X) similarly. On the formation of the peptide linkage, dicyclohexyl carbodiimide gave generally good results, but in the course of saponification of ethyl carbobenzoxyglycyl-L-prolyl-L-leucinate (VIII), a small amount of non-saponifiable substance remained. This material may be the addition product of carbodiimide with carbobenzoxy peptide, as was reported by other workers[16,17].

Carbobenzoxyglycyl-L-prolyl-L-leucine (IX) was hard to dissolve in most usual organic solvents and exhibited a marked contrast to the great solubility of carbobenzoxy-L-prolyl-L-leucylglycine (III). Both the free tripeptides, L-prolyl-L-leucylglycine (IV) and glycyl-L-prolyl-L-leucine (X), were easily soluble in glacial acetic acid and water, sparingly soluble in hot methanol containing some water and in hot dimethylformamide, and insoluble in other usual organic solvents. These solubility behaviors rather limited the experimental conditions for polycondensation. The tripeptide, glycyl-L-prolyl-L-leucine (X), contained one molecule of firmly bound crystallization water, and it could not be removed by heating over phosphorus pentoxide at 135°C(2 mmHg) for six hours. The significance of this water will be discussed in a later section.

Polycondensation of tripeptides. — In the polycondensation of L-prolyl-L-leucylglycine (IV) by the carbodiimide and the tetraethyl pyrophosphite procedures or in the polycondensation of its *p*-nitrothiophenylester, the most promising result was obtained by pyrophosphite. The most favorable molar ratio of this reagent to the tripeptide was about 1.5~2, the amount of consumption of the reagent by inevitable contamination of water being taken into consideration, and excess or insufficiency of reagent reduced the molecular

8) R. A. Boissonas and I. Schumann, *Helv. Chim. Acta*, **35**, 2229 (1952).
9) J. Noguchi and T. Hayakawa, *J. Am. Chem. Soc.*, **76**, 2846 (1954).
10) S. G. Waley, *J. Chem. Soc.*, **1955**, 517.
11) V. Bruckner, M. Szekerke and J. Kovács, *Naturwiss.*, **43**, 107 (1956).
12) C. Ressler and V. du Vigneaud, *J. Am. Chem. Soc.*, **76**, 3107 (1954).
13) R. Schwyzer, B. Iselin, W. Rittel and P. Sieber, *Helv. Chim. Acta*, **39**, 872 (1956).
14) G. W. Kenner and J. M. Turner, *Chem. and Ind.*, **1955**, 602.
15) J. R. Vaughan, Jr. and R. L. Osato, *J. Am. Chem. Soc.*, **73**, 5553 (1951).
16) J. C. Sheehan and G. P. Hess, ibid., **77**, 1067 (1955); ibid., **78**, 1367 (1956).
17) H. Zahn and J. F. Diehl, *Angew. Chem.*, **69**, 135 (1957).

weight of polypeptide formed. Tetraethyl pyrophosphite is very sensitive to moisture and the estimation of its purity was difficult, hence, the reproducibility of the result was rather inadequate.

The use of carbodiimide for polycondensation of L-prolyl-L-leucylglycine (IV) was limited by the low solubility of the tripeptide in inert solvents such as dimethylformamide at room temperature. At higher temperature, although the tripeptide dissolved in dimethylformamide, none of the free carboxyl group was detected in the product, where the addition reaction of the reagent to the end-carboxyl group may proceed in the course of polycondensation.

The *p*-nitrophenyl thioester procedure for polycondensation was also limited by the lack of available solvent, quite different from the method of Kenner[14] for cyclic peptides. In this reaction, it was considered to be reasonable that anhydrous organic solvents were preferred to water, because water would not be expected to dissolve the high molecular-weight polypeptide. Equivalent triethylamine was used for the separation of hydrogen bromide, but the reacting solution became colored markedly and the difficulty of separation of sulfur-containing compound from the product gave an undesirable result.

The polycondensation reaction by the pyrophosphite procedure was carried out at 100°, so it was supposed that the thermal side reaction might occur in the process and that equilibrium might exist between peptide linkages of the polycondensate. If the peptide-bond exchange or chain degradation occurs to any appreciable degree, the sequence of amino acid residues in the polypeptide chain would no longer retain that of the starting tripeptide. In order to clarify these questions, the nature of end amino acid residues and the content of phosphorus were determined on the products from the tripeptide(IV). After the complete hydrolysis of the polypeptide, proline, leucine and glycine were determined by paper chromatography. No appreciable amount of phosphorus was detected. From the amino nitrogen determination by the manometric van Slyke method, the free NH_2 group could not be detected either, so the *N*-terminal residue was assumed to be wholly L-proline. Determination of *C*-terminal amino acid residue of this polypeptide by hydrazinolysis[18] gave only DNP-glycine. All of these results indicated that no change in the sequence of amino acid residues has occurred during the course of the polycondensation by tetraethyl pyrophosphite.

The position of prolyl residue in tripeptide should have some influences on the peptide chain configuration which would affect the reactivity of either or both end groups in the polycondensation process. This factor must also depend upon the nature of end amino acid residues. To see these effects, the polycondensation reactions of both the tripeptides, L-prolyl-L-leucylglycine (IV) and glycyl-L-prolyl-L-leucine (X), were compared in parallel runs under the same conditions by the tetraethyl pyrophosphite procedure. The comparison indicated that the former gave a higher molecular weight product. This result differs from the expectation that the *N*-terminal glycyl residue on the tripeptide (IV) would be more reactive than the *N*-terminal prolyl residue on the tripeptide (X). Taking into consideration the results of Rydon[19], the latter peptide may be oriented in a rather rigid chain configuration.

Poly-(glycyl-L-prolyl-L-leucyl), prepared from both the tripeptides, IV and X, contained very firmly bound water, the amount of which was one mole per tripeptide unit. The tripeptide, glycyl-L-prolyl-L-leucine (X), also contained one molecule of crystallization water, as mentioned above. In addition, many other workers[19,20] have also reported the presence of crystallization water in the oligopeptides containing glycyl-prolyl residues. These facts suggest that the glycyl-prolyl linkage always has an ability to fix water molecule very firmly. If this hydration ability is general, this factor also may play a major role in the configuration of polypeptide chain of gelatin and collagen in addition to the other effects described in the first section.

The highest molecular weight fraction of the products by the pyrophosphite procedure had a molecular weight of about 6000, and this material was rather brittle and all efforts to make it into film or fiber form failed. This material showed three peaks (12.0; 6.6; 4.0 Å) in the X-ray diffraction chart by using Norelco diffractometer.

18) S. Akabori et al., This Bulletin, **29**, 507 (1956).
19) H. N. Rydon and P. W. G. Smith, *J. Chem. Soc.*, **1956**, 3642.
20) J. R. Vaughan and J. A. Eichler, *J. Am. Chem. Soc.*, **76**, 2474 (1954); N. C. Davis and E. L. Smith, *J. Biol. Chem.*, **200**, 373 (1953).

Experimental

Ethyl carbobenzoxy-L-leucylglycinate (I).—To a solution of 2.65 g. of carbobenzoxy-L-leucine[21] and 2.3 g. of dicyclohexyl-carbodiimide[22] in 20 ml. of tetrahydrofuran was added a solution of 1.67 g. of ethyl glycinate hydrochloride and 1.25 g. of triethyl amine in 10 ml. of tetrahydrofuran. The mixture was allowed to stand at room temperature for 4 hours. A small amount of acetic acid was added to the reaction mixture and after 30 minutes, the separated urea derivative was filtered off. The mother liquor was evaporated to dryness under reduced pressure and the residue was dissolved in ethyl acetate. The solution was washed with three portions of sodium bicarbonate solution, one portion of 0.2 N hydrochloric acid and water successively, and dried over magnesium sulfate. Evaporation of the solvent gave a viscous syrup, from which crystals separated out immediately on the addition of some petroleum ether. The colorless needles obtained were recrystallized from aqueous ethanol; m.p. 102~103°C and $[\alpha]_D^{18}$ −26.94° (c 5.01, ethanol). The yield was 2.65 g. (75%). Anderson et al.[23] reported a yield of 80%, m.p. 104~105°C and $[\alpha]_D^{25}$ −26.4°, and also yield of 65%, m.p. 102~103°C and $[\alpha]_D^{25}$ −27.2°. Bergmann[24] reported m. p. 103~104°C.

Ethyl carbobenzoxy-L-prolyl-L-leucylglycinate (II).—This substance was prepared from carbobenzoxy-L-proline[3] and I according to the description of du Vigneaud et al.[12] by the mixed anhydride procedure using isovaleryl chloride in 92% yield; m. p. 147~148°C, $[\alpha]_D^{18}$ −80° (c 2.5, ethanol). These values were identical with those reported previously[12].

Carbobenzoxy-L-prolyl-L-leucylglycine(III).—This substance was prepared from II according to the description of du Vigneaud et al.[12] in 91.5% yield; m. p. 163~164°C, $[\alpha]_D^{22.5}$ −85.0° (c 3.23, ethanol). These values were identical with those reported previously[12].

L-Prolyl-L-leucylglycine (IV).—To a solution of 6 g. of III in 150 ml. of methanol containing a small amount of glacial acetic acid was added 4 g. of palladium-charcoal, and hydrogen was introduced into the suspension for three hours under stirring. The catalyst was filtered off and washed thoroughly with distilled water, and then the combined filtrate was concentrated under reduced pressure. The residual syrup was dried by the addition of benzene followed by evaporation under reduced pressure. The remaining white powdery mass was dissolved in 10% methanolic acetic acid and reprecipitated with hot ethyl acetate. The yield of the colorless needles which were obtained was 3.5g. (84.5%); m.p. 220°C (decomp.), $[\alpha]_D^{20}$ −83.7° (c 3.43, glacial acetic acid).

Anal. Found: C, 54.54; H, 8.01; N, 14.80; mol. wt. 290*. Calcd. for $C_{13}H_{23}O_4N_3$: C, 54.75; H, 8.13; N, 14.73%; mol. wt. 285.3.

Paper chromatography showed this tripeptide to have only one spot, which turned yellow with ninhydrin, with an R_f value of 0.74 in *n*-butanol: water: acetic acid (4:1:1). The pK' values, 4.0 and 9.5, of the tripeptide were determined by the method of Parke and Davis[25].

Carbobenzoxy-L-prolyl-L-leucylglycine *p*-nitrophenyl-thioester (V).—A solution of 1.85 g. of III in 5 ml. of dimethylformamide was mixed with a solution of 0.45 g. of triethylamine in 5 ml. of ethylacetate and the mixture was cooled to −5°C. A solution of 0.48 g. of ethyl chlorocarbonate in ethyl acetate was then added dropwise into the reaction mixture at −5°C under stirring. After ten minutes a solution of 0.69 g. of *p*-nitrothiophenol and 0.45 g. of triethylamine in 10 ml. of ethyl acetate was added into the reaction mixture, which was allowed to stand for one hour at room temperature, when the dark red color of the mixture vanished gradually. The reacted solution was washed with dilute hydrochloric acid and dried over magnesium sulfate. Evaporation of the solvent followed by addition of petroleum ether, gave colorless crystals. The yield was 2.22 g. (90%). After recrystallization from aqueous ethanol, this substance melted at 137~138.5°C; wt. 1.9 g.

Anal. Found: C, 58.13; H, 5.99; N, 9.99. Calcd. for $C_{27}H_{32}O_7N_4S$: C, 58.25; H, 5.75; N, 10.08%.

L-Prolyl-L-leucylglycine *p*-nitrophenyl-thioester hydrobromide (VI).—A solution of 1.75 g. of V in 5 ml. of glacial acetic acid was saturated with dry hydrogen bromide. After one hour at room temperature, the solution was concentrated under reduced pressure and the residual syrupy mass was reprecipitated from glacial acetic acid by the addition of dry ether. The yield of the white powder was 1.57 g. (97%); m. p. 170°C (decomp.). This material was very hygroscopic and its aqueous alkaline solution was dark red. Analytical value of bromine determined by the Volhard method was 94% of the theoretical, but further purification of the substance was unsuccessful.

Carbobenzoxyglycyl-L-proline (VII).—This substance was prepared from carbobenzoxyglycyl chloride and L-proline according to the description of Bergmann et al.[26] in 77% yield; m. p. 155°C. Rydon[19] reported m.p. 155°C and Bergmann reported m. p. 156°C.

Ethyl carbobenzoxyglycyl-L-prolyl-L-leucinate (VIII).—This substance was prepared both

21) M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, **115**, 593 (1936).
22) E. Schmidt, F. Hitzler and E. Lahde, *Ber.*, **71**, 1933 (1938).
23) G. W. Anderson et al., *J. Am. Chem. Soc.*, **74**, 5307, 5309 (1952).
24) M. Bergmann, L. Zervas and J. S. Fruton, *J. Biol. Chem.*, **111**, 225 (1935).

* The molecular weight was determined by the end-carboxyl titration using sodium methoxide in dimethylformamide: J. S. Fritz and N. M. Lisicki, *Anal. Chem.*, **23**, 589 (1951); M. Sela and A. Berger, *J. Am. Chem. Soc.*, **77**, 1893 (1955).
25) T. V. Parke and W. W. Davis, *Anal. Chem.*, **26**, 642 (1954).
26) M. Bergmann et al., *Ber.*, **65**, 1192 (1932); *Z. physiol. Chem.*, **212**, 72 (1932).

by carbodiimide and by mixed anhydride procedures, but all efforts for crystallization failed in every case. (a) Carbodiimide procedure. To a solution of 9.3 g. of VII and 6.8 g. of dicyclohexyl-carbodiimide in 70 ml, of tetrahydrofuran was added a suspension of 7.5 g. of ethyl L-leucinate hydrochloride and 3.82 g. of triethylamine in 50 ml. of tetrahydrofuran, and the mixture was allowed to stand overnight. The reacted mixture was treated in the same manner as in the case of I and 13 g. of syrupy mass was obtained. (b) Mixed anhydride procedure. Two grams of VII, 0.7 g. of isovaleryl chloride and 1.3 g. of ethyl L-leucinate were treated in the same manner as in the case of II in tetrahydrofuran and 2.3 g. of the syrupy mass was obtained.

Carbobenzoxyglycyl-L-prolyl-L-leucine (IX).—To a solution of 13 g. of VIIIa in 40 ml. of acetone was added 16 ml. (1.1 equivalent) of 2 N sodium hydroxide. After 2.5 hours at room temperature (10°C) the solution was acidified and the separated oil was extracted with ethyl acetate. The ethyl acetate solution was extracted again with aqueous sodium bicarbonate, and the aqueous layer was acidified. A white crystalline solid separated, which was filtered off. After recrystallization from a large amount of aqueous methanol this solid gave 8.4 g. (66% based on VII) of the colorless plates; m. p. 201~202°C, $[\alpha]_D^{18}$ −62.7° (c 1.4, dimethyl formamide).

Anal. Found: C, 60.51; H, 6.75; N, 10.08. Calcd. for $C_{21}H_{29}O_6N_3$; C, 60.13; H, 6.97; N, 10.02%.

Glycyl-L-prolyl-L-leucine (X).—To a solution of 2.7 g. of IX in 100 ml. of methanol containing a small amount of glacial acetic acid was added 2 g. of palladium-charcoal, and hydrogen was introduced into the suspension for two hours under stirring. Then, the catalyst was filtered off and washed with water. The combined filtrate was concentrated to dryness under reduced pressure, and colorless needles of 1.55 g. were obtained in 85% yield. After recrystallization from aqueous methanol, the substance decomposed at 180~190°C; $[\alpha]_D^{9.5}$ −124° (c. 2.16, glacial acetic acid), $[\alpha]_D^{20.5}$ −125.1° (c 3.1, water).

Anal. Found*: C, 52.43; H, 8.15; N, 13.53. Calcd. for $C_{13}H_{23}O_4N_3 \cdot H_2O$: C, 51.47; H, 8.37; N, 13.85%. Found**: C, 54.61; H, 7.18; N, 15.00. Calcd. for $C_{13}H_{23}O_4N_3$: C, 54.75; H, 8.13; N, 14.73%. Paper chromatography showed this tripeptide to have only one spot, which turned yellow with ninhydrin, with an Rf value of 0.72 in *n*-butanol: water: acetic acid (4 : 1 : 1).

Polycondensation of L-prolyl-L-leucylglycine by the pyrophosphite procedure.—The suspension of 2.74 g. of IV and 4.0 g. of tetraethylpyrophosphite (1.5 equivalent against IV) in 7 ml. of diethylphosphite was sealed in a glass tube and heated in a boiling water bath. After about 5 minutes the tripeptide dissolved clearly in the solvent, and after about half an hour another slight precipitate appeared. The heating was continued for another hour, but no increase was observed in the amount of the precipitate. After cooling to room temperature 2 ml. of water was added into the reaction mixture in order to hydrolyse the excess reagent and active centers in the product. Removal of the diethylphosphite and water under reduced pressure left the polycondensation product as a white amorphous solid, which was treated with ethanol and ether for pulverization. This product was soluble in hot ethanol and partially soluble in hot methanol. After the hot methanol-soluble part was extracted out, the residue was dissolved in hot ethanol and fractionated with ether into three parts; namely, the first precipitate (Fr-1), the second precipitate (Fr-2) from the first mother liquor, and the residual part (Fr-3) which was obtained from the second mother liquor after removal of the solvent and the treatment with ether. The hot methanol extract was concentrated to dryness and the residue was pulverized by adding ether (Fr-4). The first fraction (Fr-1) was further fractionated in two parts, (Fr-1A) and (Fr-1B), using dimethylformamide and ether. The yields and the molecular weights of these fractions are given in Table I.

TABLE I

YIELD OF EACH FRACTION AND ITS MOLECULAR WEIGHT OF THE POLYCONDENSATE OF L-PROLYL-L-LEUCYLGLYCINE BY THE PYROPHOSPHITE PROCEDURE

Fraction number	Yield (mg.)	Mol. wt.*
Fr-1	480	—
Fr-1A	90	6600
Fr-1B	340	4200
Fr-2	450	3000
Fr-3	440	2000
Fr-4	615	1200
Total	1,985 mg. (77% based on IV)	

* These values were determined by the end-carboxyl titration using sodium methoxide in dimethylformamide: M. Sela and A. Berger, *J. Am. Chem. Soc.*, **77**, 1893 (1955).

The polypeptide (Fr-1B) was soluble in glacial acetic acid, pyridine, dimethylformamide and hot ethanol, and insoluble in water, benzene, chloroform and ethyl acetate; m. p. 190°C (decomp), $[\alpha]_D^{20}$ −104° (c 5.3, dimethylformamide). This material showed no biuret reaction.

Anal. Found: C, 54.81; H, 7.82; N, 14.70. Calcd. for $(C_{13}H_{21}O_3N_3 \cdot H_2O)_n$: C, 54.75; H, 8.13; N, 14.73%.

The phosphorus content of this material was determined by the method of Fiske[27], but it was only less than 0.01%. No amino nitrogen was detected by the manometric van Slyke method. This material was hydrazinolysed[18] with

* This substance was dried at 118°C (2 mm.) for 6 hours.

** This substance was dried at 138°C (2 mm.) for 6 hours. In this state it was strongly hygroscopic and easily returned to the initial state by being carelessly allowed to stand in the air.

27) C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

anhydrous hydrazine for 12 hours and the reaction mixture was dinitrophenylated. Thus, only one spot corresponding to DNP-glycine was detected by paper chromatography[28] from the acidic part of the reaction mixture, in which all the *C*-terminal amino acid of the polypeptide must be included.

The lower molecular weight fractions (Fr-3, Fr-4) were treated once more with tetraethyl-pyrophosphite in the same manner as described above, but no molecular weight increase was observed.

Polycondensation of L-prolyl-L-leucylglycine by the carbodiimide procedure.—One gram of dicyclohexylcarbodiimide and 1.07 g. of IV were suspended in 5 ml. of dimethylformamide and the suspension was sealed in a glass tube, but no change in the appearance of this suspension was observed at room temperature. When the mixture was heated at 70°, it changed to a clear solution. After 3 hours the solution was cooled to room temperature and the dimethylformamide was removed under reduced pressure. The residual syrup was treated with ethanol and ether, and 1.0 g. of crystalline product was obtained. However, the separation of polypeptide from the product was unsuccessful and no free carboxyl group was detected in this material by the sodium methoxide titration.

Polycondensation of L-prolyl-L-leucylglycine *p*-nitrophenylthioester.—The solution of 500 mg. of VI and an equivalent of triethylamine in 2 ml. of dimethylformamide was sealed in a glass tube and heated at 60° for 15 minutes. After standing at room temperature for 18 hours the resulting dark red solution changed to light yellow on the addition of a small amount of hydrochloric acid, and then excess of water was added to the solution. The precipitate formed was filtered off and dissolved in glacial acetic acid. Some hydrogen peroxide was added to the solution and the crystals which separated were filtered off. The mother liquor was concentrated under reduced pressure, and then the residual mass was extracted with hot ethanol. The extract was concentrated to dryness and the residue was treated twice in the same way as described above. The last amorphous residue was purified with dimethylformamide and ether, and about 100 mg. of white powdery substance was obtained. The molecular weight of this fraction (1150) was determined by end-carboxyl titration, but no analytical data were determined.

Parallel runs of the polycondensation reaction of L-prolyl-L-leucylglycine (IV) and of glycyl-L-prolyl-L-leucine (X).—The tripeptides IV and anhydrous X were treated in parallel runs under the same condition by the pyrophosphite procedure. The results are listed in Table II.

TABLE II

COMPARISON OF THE POLYCONDENSATION PRODUCTS OF THE TWO TRIPEPTIDES IV AND X

Tripeptide	mg.	Diethyl-phosphite ml.	Tetraethyl-pyrophosphite mg.	Yield mg.	Yield %	Mol. wt.
IV	400	3.0	700	250	60	3000
X	400	3.0	700	130	35	1900
IV	400	2.5	1300	300	80	550
X	400	2.5	1300	170	45	500

Summary

The two tripeptides, L-prolyl-L-leucylglycine (IV) and glycyl-L-prolyl-L-leucine (X), were synthesized, and IV was subjected to polymerization by the tetraethyl pyrophosphite, carbodiimide and *p*-nitrophenyl thioester methods, in which the most promising result was obtained by the pyrophosphite method. The highest molecular weight of the polypeptide which was obtained was about 6000 and the repetition of the original tripeptide unit in the polypeptide was confirmed by the end groups determination. The polycondensation reactions of both the tripeptides, IV and X, were compared in parallel runs by the tetraethyl pyrophosphite procedure, and the comparison showed that IV gave a better yield and a higher molecular weight product than X. X and poly-(L-prolyl-L-leucyl-glycyl), which was obtained from IV, contained firmly bound water in spite of the fact that IV contained no crystallization water.

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28) A. L. Levy, *Nature*, **174**, 126 (1954).