The Enzymic Hydroxylation of Protocollagen Models

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1. Synthetic polymers of L-prolyl-L-prolylglycine of defined chain length, (Pro-Pro-Gly)_n, were found to be substrates for the enzyme protocollagen-proline hydroxylase, with optimum chain length $n=5$. Boiling the polymer (Pro-Pro-Gly)₁₅ increased its activity as a substrate but had no effect on (Pro-Pro-Gly)₅. 2. Protection of both or one of the *N*- and *C*-terminal groups made (Pro-Pro-Gly)₃ a better substrate, and collagenase digestion of hydroxylated *tert*.-pentylloxycarbonyl-(Pro-Pro-Gly)₃ benzyl ester indicated that the central prolyl residues were the major points of hydroxylation. 3. The results suggest that the long-chain peptides are optimum substrates but that a triple-stranded structure is inhibitory for hydroxylation.

Hydroxyproline in collagen is synthesized biologically by the hydroxylation of proline residues incorporated into the large polypeptide precursor protocollagen (Prockop & Kivirikko, 1967). The protocollagen can be obtained from chick embryos in which the hydroxylation is inhibited with $\alpha\alpha'$ -bipyridyl and can be used as a substrate for the hydroxylating enzyme protocollagen-proline hydroxylase. The enzyme purified from chick-embryo homogenate hydroxylates the proline residues not only in protocollagen but also in synthetic polypeptides (Pro-Gly-Pro)_n, with ill-defined molecular weights (Kivirikko & Prockop, 1967). The tripeptide Gly-Pro-Pro and poly-L-proline are not hydroxylated by the enzyme (Kivirikko & Prockop, 1967).

Sakakibara, Kishida, Kikuchi, Sakai & Kakiuchi (1968a) synthesized sequential polypeptides (Pro-Pro-Gly)_n with defined molecular weights by step-wise addition of AOC*-Pro-Pro-Gly to the Merrifield resin. The products (Pro-Pro-Gly)_n showed some properties that were not observed with the above ill-defined polymers. First, the peptides (Pro-Pro-Gly)_n with n values above ten were less soluble in water than the analogues with n values under ten. Secondly, a temperature-dependent transition in optical rotation or in apparent molecular weight was observed with (Pro-Pro-Gly)_n ($n > 10$), but was not observed with the products with n less than ten (Sakakibara *et al.* 1968a). At a temperature lower than the transition point, the apparent molecular weight of (Pro-Pro-Gly)_n ($n > 10$) was three times the calculated value. The transition

Abbreviations: AOC, *tert*.-pentylloxycarbonyl-; -OBzl, benzyl ester.

may be ascribed to the conformational change between single- and triple-stranded structures, as observed with collagen. These polypeptides with defined molecular weights were subjected to the protocollagen-proline hydroxylase action, and the effects of chain length and conformation of the peptides on the hydroxylation were examined.

With the short (Pro-Pro-Gly)_n peptides ($n < 5$) the longer peptides were found to be more rapidly hydroxylated, and the effect of substitution at terminal groups of (Pro-Pro-Gly)₃ was therefore examined.

The position of hydroxylation was confirmed on the enzymic hydroxylation product of AOC-(Pro-Pro-Gly)₃-OBzl.

A part of the work has been published in a preliminary communication (Kikuchi, Fujimoto & Tamiya, 1969).

MATERIALS AND METHODS

Synthetic polypeptides (Pro-Pro-Gly)_n. Synthesis of the polypeptides (Pro-Pro-Gly)_n was carried out by peptide coupling on the Merrifield resin as described by Sakakibara *et al.* (1968a). AOC-Pro-Pro was coupled to glycine bound to the resin with dicyclohexylcarbodi-imide. After the removal of AOC group with HCl in acetic acid, coupling cycles with the protected tripeptide AOC-Pro-Pro-Gly were repeated until the resin-linked peptide had a desired length. The peptides were removed from the resin with anhydrous HF (Sakakibara, Shimonishi, Kishida, Okada & Sugihara, 1967; Sakakibara, Kishida, Nishiwaza & Shimonishi, 1968b) and purified by gel filtration or dialysis.

AOC-(Pro-Pro-Gly)₂-OBzl. An oily preparation of AOC-Pro-Pro-Gly-OBzl (6 m-moles), synthesized as described by Sakakibara & Inukai (1966), was treated with

trifluoroacetic acid (4.5 ml., 60 m-moles) at room temperature for 15 min. The excess of trifluoroacetic acid was then evaporated off *in vacuo*, and the remaining oil was stored over NaOH *in vacuo* overnight. The residue was dissolved in chloroform (5 ml.), neutralized with triethylamine and allowed to react with AOC-Pro-Pro-Gly *p*-nitrophenyl ester (2.26 g., 4.5 m-moles) (Sakakibara & Inukai, 1966). After standing for 4 days at room temperature, the solution was diluted with chloroform (50 ml.) and washed with *m*-NH₃ (total 200 ml.), 0.5 M-HCl (60 ml.) and water (40 ml.). After the chloroform solution had been dried over anhydrous Na₂SO₄ (approx. 30 g.), the solvent was evaporated off *in vacuo*. The residue was dissolved in ethyl acetate (10 ml.) and crystallized by the addition of light petroleum (b.p. 50–90°) (approx. 5 ml.), yield 2.01 g. (61.5%), m.p. 147–149°. Recrystallization from ethyl acetate–chloroform (6/1, v/v) gave 1.81 g. (55.4%) of the product, m.p. 149.5–150° (Found: C, 61.0; H, 7.3; N, 11.5. Calc. for C₃₇H₅₂N₆O₉: C, 61.3; H, 7.2; N, 11.6%).

AOC-(Pro-Pro-Gly)₃-OBzl. AOC-(Pro-Pro-Gly)₂-OBzl (1.45 g., 2 m-moles) was treated with trifluoroacetic acid (1.5 ml., 20 m-moles) and allowed to react with AOC-Pro-Pro-Gly *p*-nitrophenyl ester in chloroform as described above. After standing for 2 days, the reaction mixture containing colloidal product was diluted with ethyl acetate and the product was precipitated by the addition of dry ether. Recrystallization from small amounts of ethanol, ethyl acetate and dry ether gave 1.58 g. (79.5%) of the product, m.p. 143.5–147.5° (Found: C, 58.9; H, 7.4; N, 12.5. Calc. for C₄₉H₅₉N₁₀O₁₂·H₂O: C, 59.1; H, 7.2; N, 12.6%).

(Pro-Pro-Gly)₃-OBzl. AOC-(Pro-Pro-Gly)₃-OBzl (98 mg., 0.1 m-mole) was treated with trifluoroacetic acid (1.2 ml., 1.6 m-moles) and the excess of trifluoroacetic acid was evaporated off. The residue was dissolved in water (5 ml.) and remaining trifluoroacetic acid was removed with a column (1.2 cm. × 7 cm.) of Dowex 1 (X4; acetate form). The aqueous eluate was concentrated *in vacuo* and then freeze-dried to give an amorphous powder (91 mg.).

AOC-(Pro-Pro-Gly)₃. AOC-(Pro-Pro-Gly)₃-OBzl (98 mg., 0.1 m-mole) in methanol (10 ml.) was subjected to hydrolysis in the presence of palladium black catalyst, which was removed by filtration when the reaction was over. The filtrate was concentrated and the residue was dissolved in water (5 ml.). The solution was freeze-dried to give an amorphous powder (87 mg.).

The above synthetic reactions, except for the solid-phase synthesis, were followed by t.l.c. (Morita & Haruta, 1963).

Procollagen-proline hydroxylase. The enzyme was purified from chick-embryo homogenate as described by Kivirikko & Prockop (1967). Chick embryos (12-day-old) were homogenized in 0.01 M-KCl (1 ml./g. of embryo) in a Waring Blender. After centrifugation of the homogenate at 15000g for 30 min. the supernatant was fractionated by the addition of solid (NH₄)₂SO₄. The fraction, soluble at 30% saturation and insoluble at 65% saturation, was collected and dissolved in 0.02 M-tris-HCl buffer containing 0.05 M-KCl (0.2 ml./ml. of the homogenate) and dialysed against cold 0.02 M-tris-HCl buffer, pH 7.2, containing 0.05 M-KCl (50 ml./ml. of sample solution) for 15 hr. The dialysed preparation was diluted with 0.05 M-KCl to a protein concentration of 15 mg./ml. and added with 26 ml. of calcium phosphate gel suspension [11.3 mg. of solid (Calbiochem, Los Angeles, Calif., U.S.A.)/ml.]/1000 mg. of protein. The gel was separated by centrifugation at

2500g for 15 min. (gel 1+2). Gels (3), (4) and (5) were prepared similarly by stepwise addition of each 13 ml. of gel suspension to the preceding supernatant. The protein was eluted from the gel in two steps with (A) 0.02 M- and (B) 0.15 M-potassium phosphate buffer, pH 7.2 (each 2 ml./ml. of the gel suspension used). The eluates were added with solid (NH₄)₂SO₄ to 50% saturation and centrifuged at 15000g for 30 min. The loose pellets obtained were dissolved in 0.02 M-tris-HCl buffer, pH 7.2, containing 0.05 M-KCl (0.1 ml./ml. of the eluate) and dialysed against the same buffer solution (200 ml./ml. of sample solution) for 15 hr.

Activity of the enzyme preparations. The specific activity of the procollagen-proline hydroxylase preparations was determined by the hydroxylation of reduced and carboxymethylated collagen of *Ascaris* cuticle (Fujimoto & Prockop, 1968) as substrate. The reaction conditions and the assay procedure are shown below. Protein concentration of the enzyme preparations was measured by the extinction at 225 nm. The highest specific activity was observed usually with fraction 4-B or 5-B, which was used for the experiments described below.

Enzymic reaction and its assay. The enzymic reaction was carried out in a mixture (4 ml.) containing the reduced and carboxymethylated *Ascaris*-cuticle collagen (25 μg./ml.) or synthetic polypeptide (125–500 μg./ml.) and the procollagen-proline hydroxylase (1.5–2.0 mg. of enzyme protein/ml.) in 0.04 M-FeSO₄–0.5 mM-α-oxoglutarate–2 mM-ascorbic acid–0.05 M-tris-HCl buffer, pH 7.8 at 25°. In some cases the substrate solution in 0.2 M-tris-HCl buffer was heated at 100° for 30 min. just before the reaction. The reaction mixture was shaken (60 strokes/min.) at 37° in the atmosphere. After reaction the mixture was treated with an equal volume of 12 M-HCl and heated in a sealed glass tube at 110° for 15–20 hr. The dried residues of the hydrolysate were dissolved in 1.2 ml. of water and the hydroxyproline content in a 1 ml. portion was assayed by the Prockop method (Kivirikko, Laitinen & Prockop, 1967).

RESULTS

Chain length and hydroxyproline formation. Fig. 1 shows the time-course of the enzymic hydroxylation carried out with (Pro-Pro-Gly)_{*n*}, *n* = 3, 5 and 15 (each 250 μg./ml.). (Pro-Pro-Gly)₅ was most rapidly hydroxylated among (Pro-Pro-Gly)_{*n*}, *n* = 3, 5 and 15. The optimum chain length was investigated by treating substrates, (Pro-Pro-Gly)_{*n*}, *n* = 1, 3, 5, 10, 15 and 20 (each 250 μg./ml.), with the enzyme (1.8 mg./ml.) for 60 min. and assaying the hydroxyproline formation (Fig. 2). There was a maximum at *n* = 5. The tripeptide Pro-Pro-Gly was hydroxylated, whereas Gly-Pro-Pro has been reported not to be hydroxylated (Kivirikko & Prockop, 1967).

Effect of boiling the substrate solution. The boiling procedure was carried out at 100° for 30 min. just before the enzymic hydroxylation. Boiled (Pro-Pro-Gly)₁₅ was more easily hydroxylated than unboiled (Fig. 3a), whereas boiled and unboiled (Pro-Pro-Gly)₅ showed no difference in hydroxyproline formation (Fig. 3b).

Fig. 1. Time-course of the reaction. (Pro-Pro-Gly)_n, $n=3, 5$ and 15 (each 1 mg.), and enzyme (7 mg. of protein) were incubated at 37° in 4 ml. of reaction mixture containing 0.04 mM-FeSO_4 , $0.5\text{ mM-}\alpha\text{-oxoglutarate}$, $2\text{ mM-ascorbic acid}$ and 0.05 M-tris-HCl buffer, $\text{pH}7.8$. $\diamond$, (Pro-Pro-Gly)₃; $\circ$, (Pro-Pro-Gly)₅; $\triangle$, (Pro-Pro-Gly)₁₅.

Fig. 2. Effect of chain length on the hydroxylation. Substrates, (Pro-Pro-Gly)_n (each 1 mg.), and enzyme (7 mg. of protein) in 4 ml. were incubated at 37° for 60 min. in a similar reaction mixture to that of Fig. 1.

Various amounts of substrates, boiled and unboiled, were treated with the enzyme for 60 min. Fig. 4(a) shows the results obtained with (Pro-Pro-Gly)₁₅. The hydroxyproline formation with

Fig. 3. (a) Effect of boiling procedure of (Pro-Pro-Gly)₁₅ solution on the hydroxylation. A solution of (Pro-Pro-Gly)₁₅ (2 mg./ml. of 0.2 M-tris-HCl buffer, $\text{pH}7.8$) was heated at 100° for 30 min. just before the reaction. The peptide and the enzyme (7 mg. of protein/ 4 ml.) were incubated at 37° in a similar reaction mixture to that of Fig. 1. The unboiled peptide was hydroxylated at the same time. $\diamond$, Boiled (Pro-Pro-Gly)₁₅; $\circ$, unboiled (Pro-Pro-Gly)₁₅. (b) Effect of the boiling procedure of (Pro-Pro-Gly)₅ solution on the hydroxylation. A solution of (Pro-Pro-Gly)₅ was heated as described in (a). The boiled and unboiled (Pro-Pro-Gly)₅ (each 1 mg./4 ml.) were incubated with the enzyme (7 mg. of protein/ 4 ml.). $\diamond$, Boiled (Pro-Pro-Gly)₅; $\circ$, unboiled (Pro-Pro-Gly)₅.

Fig. 4. (a) Effect of concentration of (Pro-Pro-Gly)₁₅ on the hydroxylation. A solution of (Pro-Pro-Gly)₁₅ (2 mg./ml. of 0.2M-tris-HCl buffer, pH 7.8) was heated at 100° for 30 min. just before the reaction. Boiled and unboiled (Pro-Pro-Gly)₁₅ were treated with the enzyme (10 mg. of protein/4 ml.) at 37° for 60 min. $\diamond$, Boiled (Pro-Pro-Gly)₁₅; $\circ$, unboiled (Pro-Pro-Gly)₁₅. (b) Effect of concentration of (Pro-Pro-Gly)₅ on the hydroxylation. A solution of (Pro-Pro-Gly)₅ was heated as described in (a). Boiled and unboiled substrates were treated with the enzyme (8 mg. of protein/4 ml.) at 37° for 60 min. in a similar reaction mixture to that of Fig. 1. $\diamond$, Boiled (Pro-Pro-Gly)₅; $\circ$, unboiled (Pro-Pro-Gly)₅.

unboiled (Pro-Pro-Gly)₁₅ increased with increasing substrate concentration up to 500 $\mu\text{g./ml.}$, whereas boiled (Pro-Pro-Gly)₁₅ gave a constant high value over the range of substrate concentration examined.

Table 1. Effect of substitution of (Pro-Pro-Gly)₃ on hydroxyproline formation

The peptides (each 2.62 $\mu\text{moles/4 ml.}$; 0.66 mg. of -Pro-Pro-Gly-unit/4 ml.) were incubated with the enzyme (10 mg. of protein/4 ml.) at 37° for 60 min. in a similar reaction mixture to that of Fig. 1, and the hydroxyproline formation was assayed.

Peptide	Hydroxyproline formed ($\mu\text{g.}$)
AOC-(Pro-Pro-Gly) ₃ -OBzl	29.8
AOC-(Pro-Pro-Gly) ₃	25.4
(Pro-Pro-Gly) ₃ -OBzl	26.6
(Pro-Pro-Gly) ₃	15.4

Fig. 4(b) shows that the curve with (Pro-Pro-Gly)₅ was not affected by the boiling.

Effect of substitution at terminal groups. The peptide (Pro-Pro-Gly)₃ and its analogues protected at both or one of the *N*- and *C*-terminal groups with AOC and OBzl groups respectively were treated with the enzyme for 60 min. and the hydroxyproline formation was assayed (Table 1). The substitution on either the *N*- or the *C*-terminal group stimulated the hydroxylation. The substitution at both termini was most effective.

Position of hydroxylation in AOC-(Pro-Pro-Gly)₃-OBzl. AOC-(Pro-Pro-Gly)₃-OBzl (8.7 mg./6 ml. of reaction mixture) was treated with the enzyme for 60 min. and then separated from the enzyme by gel filtration with Sephadex G-25 (Fig. 5). The fractions containing the reaction product were freeze-dried. Three-quarters of the hydroxylated AOC-(Pro-Pro-Gly)₃-OBzl (in 0.6 ml. of 0.05M-tris-HCl buffer, pH 8.7) was digested by collagenase (0.12 mg.) at 37°. (The purified protease-free collagenase preparation was given by Dr Hayashi and Dr Nagai, Tokyo Medical and Dental University.) It is expected from the specificity of the collagenase (Nagai, Sakakibara, Noda & Akabori, 1960) that the peptide is digested into three fragments as shown in Scheme 1. After 15 hr. 2.1 μmoles of the amino group/ μmole of AOC-(Pro-Pro-Gly)₃-OBzl were detected by ninhydrin colorimetry, with glycine ethyl ester hydrochloride as the standard. The resulting mixture of acidic, neutral and basic fragments, which contain the hydroxylated fragments AOC-Pro-Hyp, Gly-Pro-Hyp and Gly-Pro-Hyp-Gly-OBzl, were separated by cellulose-powder zone electrophoresis. The washed cellulose powder was suspended in 0.1M-pyridine-acetate buffer, pH 5.0, and a slab (40 cm. $\times$ 2 cm. $\times$ 1 cm.) was prepared. Electrophoresis was carried out in the cold-room at 4° (450 v and 35–50 ma for 120 min.). The slab was cut into blocks (1 cm. thick), which were extracted with water (2.5 ml./block). The peptides in the extract were detected qualitatively as follows. A drop of

each sample was taken on a silica gel thin-layer plate, sprayed with 47% hydrobromic acid to remove the AOC group, dried at 100° and sprayed with ninhydrin. The parts containing the acidic,

Fig. 5. Gel filtration of reaction mixture on a Sephadex G-25 column (2.8 cm. $\times$ 50 cm.). Elution was carried out with 0.2M-acetic acid. Fractions of volume 10 ml. were collected. Amino acid content in the alkali hydrolysates of the 0.2 ml. portions of the fraction was measured by ninhydrin colorimetry.

neutral and basic fragments were extracted with water and filtered (total 50 ml.). The filtrate was evaporated to dryness *in vacuo* and the residue was hydrolysed with 6M-HCl (8 ml.) at 110° for 16 hr. Hydroxyproline, proline and glycine contents of each part were assayed by the Prockop method and by an automatic amino acid analyser (type JLC-5AH; Japan Electron Optics Laboratory Co., Tokyo, Japan) (Table 2). The neutral part contained 61% of hydroxyproline formed/2 μ moles of proline residues. The hydroxyproline content of the central (neutral) fragment is twice the average value in the undigested hydroxylation product, whereas the hydroxyproline contents of terminal (acidic and basic) fragments are lower.

DISCUSSION

The reaction proceeds almost linearly for up to 60 min. (Fig. 1).

The polypeptides (Pro-Pro-Gly) $_n$ with n values 10, 15 and 20 show a transition in solution in optical rotation or in apparent molecular weight at 25°, 55° and 65° respectively, ascribed to the intermolecular conformational change between triple-stranded and

Scheme 1. Expected course of digestion of hydroxylated AOC-(Pro-Pro-Gly) $_3$ -OBzl by collagenase.

Table 2. Position of the hydroxylation in AOC-(Pro-Pro-Gly) $_3$ -OBzl

AOC-(Pro-Pro-Gly) $_3$ -OBzl (8.7 mg./6 ml. of reaction mixture) was hydroxylated by the enzyme (5 mg. of protein/6 ml.) and then digested by collagenase (0.12 mg.) into three fragments, which were separated by electrophoresis. The hydroxyproline, proline and glycine contents in each fragment were assayed. These amino acid contents in hydroxylated and undigested AOC-(Pro-Pro-Gly) $_3$ -OBzl were also assayed.

Fragment	Hydroxyproline (μ g.)	Proline (μ moles)	Glycine (μ moles)	Hydroxyproline content (μ g./2 μ moles of proline residues)	Distribution of hydroxyproline (%)
(OH) AOC-Pro-Pro- (acidic)	4.5	8.5	0	1.06	11
(OH) Gly-Pro-Pro- (neutral)	29.8	10.7	4.6	5.98	61
(OH) Gly-Pro-Pro-Gly-OBzl (basic)	8.7	6.5	4.1	2.68	28
(OH) AOC-(Pro-Pro-Gly) $_3$ -OBzl (undigested)	7.2	4.9	2.4	2.94	

single-chain structures (Sakakibara *et al.* 1968a). A similar phenomenon was observed with natural collagen (Harrington & Hippel, 1961) but not with uncontrolled polymerization products (Pro-Pro-Gly)_n, which have a distribution of molecular weights (Engel, Kurtz, Katchalski & Berger, 1966). At 37°, the enzymic reaction temperature, the three-stranded structure is expected for (Pro-Gly-Pro)_n of $n=15$ and 20, and the single-chain structure for the peptides of $n=1, 3$ and 5.

Fig. 2 shows that there is a maximum in the hydroxyproline formation when the peptide chain is elongated. The amounts of peptides in reaction mixtures were the same in weight. The molar concentration of the peptides was therefore inversely proportional to the n value. The result in Fig. 2 shows that the effect of increased susceptibility of the substrates to the enzyme with increased n value exceeds that of the decrease in molar concentration of the substrates until the maximum was reached.

The hydroxyproline formation was accelerated by the substitution at the terminal groups of the substrate (Table 1). The result in Table 2 indicates that the non-terminal -Pro-Pro-Gly- unit is more easily hydroxylated than the terminal ones. The increase in the hydroxylation by elongation of the peptide chain can be ascribed to the increase of the non-terminal -Pro-Pro-Gly- unit.

The decrease in hydroxylation with increasing n values ($n=10, 15$ and 20; Fig. 2) could be ascribed to the formation of the triple-stranded structure because the unfolding by boiling stimulated the hydroxylation, as shown in Figs. 3(a) and 3(b). The greater affinity of the boiled (Pro-Pro-Gly)₁₅ for the enzyme is shown in Fig. 4(a). Boiled substrate saturated the enzyme at a concentration less than 125 µg./ml. whereas the unboiled sample did not. On the other hand, the hydroxylation of (Pro-Pro-Gly)₅, which was expected to have a single-chain conformation, was not affected by the boiling procedure (Fig. 4b).

These results suggest that the triple-stranded structure, which is formed with longer peptides, is unfavourable for the hydroxylation. There are two possibilities to explain the decrease in hydroxylation with the formation of triple-stranded structure: (1) the triple-stranded substrates are not hydroxylated, and only the monomer in equilibrium with the trimer or monomeric portion of loose triple-stranded structure is hydroxylated; (2) the apparent decrease in the substrate concentration by trimer formation is the reason for the decrease in the hydroxylation.

The same peptides (Pro-Pro-Gly)_n were hydroxylated by the hydroxylase extracted from guinea-pig granuloma (Suzuki & Koyama, 1969) and an essentially similar result to ours (Fig. 2) was obtained. Suzuki & Koyama (1969) suggested that the decrease

of hydroxyproline formation with (Pro-Pro-Gly)₂₀ should be ascribed to the insolubility of the substrate. However, the extinctions at 225 nm. of the solutions of (Pro-Pro-Gly)₅ and (Pro-Pro-Gly)₂₀ (1 mg./4 ml. of 0.05 M-tris buffer, pH 7.8) were the same after centrifugation at $1.3 \times 10^5 g$ for 60 min., and (Pro-Pro-Gly)₅ has been reported to be very soluble. Consequently, at the concentration of the substrate in our experiments the decreased solubility of (Pro-Pro-Gly)₁₅ and (Pro-Pro-Gly)₂₀ could not explain our results.

These results agree with those of Fujimoto & Prockop (1968) that reduced and carboxymethylated *Ascaris*-cuticle collagen is more easily hydroxylated after denaturation, and disagree with those of some recent papers (Hutton, Tappel & Udenfriend, 1967; Nordwig & Pfab, 1968; Kivirikko, Bright & Prockop, 1968) that boiled and unboiled procollagen are hydroxylated similarly.

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REFERENCES

- Engel, J., Kurtz, J., Katchalski, E. & Berger, A. (1966). *J. molec. Biol.* **17**, 255.
- Fujimoto, D. & Prockop, D. J. (1968). *J. biol. Chem.* **243**, 4138.
- Harrington, W. F. & Hippel, P. H. von (1961). *Advanc. Protein Chem.* **16**, 1.
- Hutton, J. J., Tappel, A. L. & Udenfriend, S. (1967). *Arch. Biochem. Biophys.* **118**, 231.
- Kikuchi, Y., Fujimoto, D. & Tamiya, N. (1969). *FEBS Lett.* **2**, 221.
- Kivirikko, K. I., Bright, H. J. & Prockop, D. J. (1968). *Biochim. biophys. Acta*, **151**, 558.
- Kivirikko, K. I., Laitinen, O. & Prockop, D. J. (1967). *Analyt. Biochem.* **19**, 249.
- Kivirikko, K. I. & Prockop, D. J. (1967). *J. biol. Chem.* **242**, 4007.
- Morita, M. & Haruta, F. (1963). *J. Chromat.* **12**, 412.
- Nagai, Y., Sakakibara, S., Noda, H. & Akabori, S. (1960). *Biochim. biophys. Acta*, **37**, 567.
- Nordwig, A. & Pfab, F. K. (1968). *Biochim. biophys. Acta*, **154**, 603.
- Prockop, D. J. & Kivirikko, K. I. (1967). *Ann. intern. Med.* **66**, 1243.
- Sakakibara, S. & Inukai, N. (1966). *Bull. chem. Soc. Japan*, **39**, 1567.
- Sakakibara, S., Kishida, Y., Kikuchi, Y., Sakai, R. & Kakiuchi, K. (1968a). *Bull. chem. Soc. Japan*, **41**, 1273.
- Sakakibara, S., Kishida, Y., Nishizawa, R. & Shimonishi, Y. (1968b). *Bull. chem. Soc. Japan*, **41**, 438.
- Sakakibara, S., Shimonishi, Y., Kishida, Y., Okada, M. & Sugihara, H. (1967). *Bull. chem. Soc. Japan*, **40**, 2164.
- Suzuki, F. & Koyama, E. (1969). *Biochim. biophys. Acta*, **177**, 154.