Acta odont scand 1971 *29:* 609—617

Keywords:
Collagen biosynthesis
Dental pulp
Rabbits

From:
The Departments of Medical Chemistry and Pharmacology, University of Helsinki, Finland

CHARACTERIZATION OF COLLAGEN BIOSYNTHESIS IN RABBIT DENTAL PULP *IN VITRO*

VELI-JUKKA UITTO
RISTO ANTILA

Collagen biosynthesis in the dental pulp was studied by measuring the rate of ^{14}C-hydroxyproline formation in vitro and assaying the activity of protocollagen proline hydroxylase (PPH). The maximal formation of ^{14}C-hydroxyproline from ^{14}C-proline in an incubation medium occurred between the pH values 7.4 and 8.2. No significant differences in the rate of collagen biosynthesis could be demonstrated between the molar and incisor pulps. The methods of the present study seem to be useful for further investigation on collagen metabolism in the dental pulp.

Collagen, the main fibrous protein of connective tissue, is composed of tropocollagen molecules. These units of collagen fibres are built up from three polypeptide chains, each containing about 1000 amino acid residues (*Veis*, 1964). In the dental pulp, as well as in other loose connective tissues, tropocollagen molecules are synthesized within fibroblasts. In calcifying tissues, such as bone and dentine, specialized cells, osteoblasts and odontoblasts are involved in the formation of collagen composing the matrix for the concomitant mineralization (*Solomons et al.*, 1960). Thus, being the most abundant protein in the body, collagen also serves as the major organic structural component in the teeth and their supporting tissues.

The biosynthesis of collagen follows the basic principles of protein synthesis in general (*Manner et al.*, 1967). However, there are some special features connected to amino acids hydroxyproline and hydroxylysine which occur almost exclusively in collagen (*Neuman & Logan*, 1950a; *Van Slyke et al.*, 1941). These components in the collagen molecule are not derived from free hydroxyproline and hydroxylysine but from proline and lysine

Received for publication, May, 6, 1971.

which are hydroxylated after being incorporated to a polypeptide precursor of collagen, called protocollagen (*Chvapil & Hurych*, 1968). The enzymes catalyzing these hydroxylations are protocollagen proline hydroxylase and protocollagen lysine hydroxylase (*Weinstein et al.*, 1969; *Halme et al.*, 1970). It is also possible that there is only one enzyme with separate sites for hydroxylations of both amino acid residues (*Kivirikko & Prockop*, 1967). The conversion of proline to hydroxyproline is the unique step in collagen formation, on which, therefore, the most convenient methods for quantitative measurement of the rate of collagen biosynthesis are based (*Kivirikko*, 1970).

The present investigation was designed to characterize the aspects of collagen biosynthesis in rabbit pulp by measuring the rate of hydroxyproline formation *in vitro* and assaying the activity of protocollagen proline hydroxylase (PPH).

MATERIAL AND METHODS

Preparation of pulps. The total number of samples included in this study was about 140 dental pulps obtained from eight rabbits of both sexes weighing from 2.2 to 3.0 kg. The pulps were removed as rapidly as possible after the rabbits had been killed by a blow on the head. Individual pulp samples, weighing from 5.5 to 36.0 mg, were placed in the incubation medium or homogenized for assays of protocollagen proline hydroxylase.

Incubation conditions. The incubation medium was composed of 20 mM N-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) buffer (Calbiochem, Los Angeles), of pH 7.4 at 37°C; 20 mM glucose; 0.05 mg/ml ampicillin (Pentrexyl, Lundbeck, Copenhagen); 1 μC ^{14}C-proline, specific activity 180 mC/mmole (New England Nuclear Corporation, Boston), all in phosphate-free Krebs-Ringer solution. Each tube contained from one to four whole pulps in a total volume of 1 ml of incubation medium. The incubations were carried out at 37°C in a metabolic shaker under atmospheric air. The incubation time ranged from 3 to 21 hours. Experiments were undertaken at various pH values (from 6.6 to 8.2) using besides HEPES buffer, 0.2 M Tris-maleate, as well. After incubation the pulps and the incubation mixture were homogenized with a Teflon and glass homogenizer at 0°C. The homogenates were dialysed against cold running tap water for 24 hours and then hydrolysed in 6 M HCl at 136°C for 6 hours.

Assay of protocollagen proline hydroxylase. Pulps were homogenized in 0.1 M KCl and 0.02 M Tris-HCl buffer (pH 7.8 at 24°C) with a Teflon and

Table I.
Concentration of collagen and total protein in rabbit dental pulps

	Molar pulps	Incisor pulps	Significance of difference
Protein mg/100 mg wet weight	5.7±3.0	5.0±2.0	ns.*
Collagen mg/100 mg wet weight	0.69±0.1	0.52±0.08	$p<0.001$
$\frac{\text{Collagen}}{\text{Total protein}} \times 100$	12.0±5.7	10.3±4.9	ns.

*ns. = nonsignificant ($p>0.05$)

glass homogenizer at 0°C. Varying amounts of the 15,000× g supernatant of pulp homogenate were incubated for 60 minutes at 37°C with ^{14}C-proline-labelled protocollagen substrate prepared as described by *Kivirikko* and *Prockop* (1967), 0.05 M Tris-HCl buffer (pH 7.8 at 24°C), 2 mM ascorbic acid, 0.5 mM α-ketoglutarate, 0.08 mM $FeSO_4$, 0.05 mg/ml catalase (Sigma, St. Louis), and 1 mM dithiothreitol (*Kivirikko & Prockop*, 1967), in a final volume of 4 ml. After incubation, the samples were hydrolysed in 6 M HCl at 136°C for 6 hours.

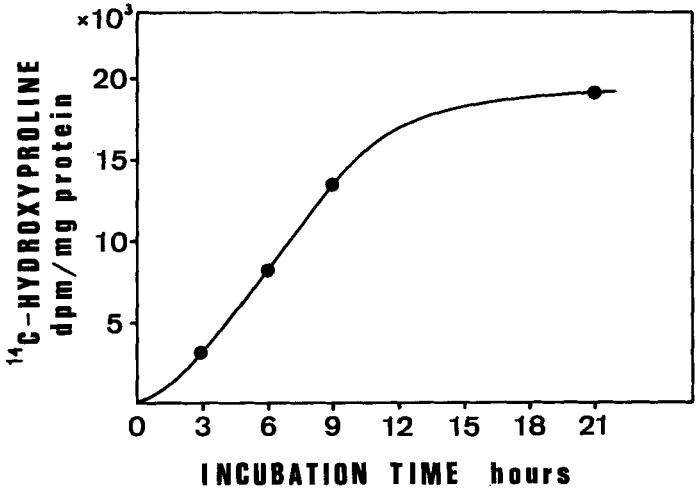

Fig. 1. Time-dependence of the formation of ^{14}C-hydroxyproline in the dental pulp in vitro. Each dot represents a sample of three molar rabbit pulps incubated at 37°C in phosphate-free Krebs-Ringer solution containing 20 mM HEPES-buffer (pH 7.4), 20 mM glucose, 0.05 mg/ml ampicillin and 1 μC^{14}C-proline.

Determination of ^{14}C-hydroxyproline and total ^{14}C-radioactivity. After hydrolysis, radioactive hydroxyproline was determined by the method of *Juva* and *Prockop* (1966) and total ^{14}C-radioactivity by the method of *Prockop* and *Ebert* (1963). Radioactivity was measured with a three-channel liquid scintillator. The observed counts were corrected for quenching and the values of radioactivity were expressed as disintegrations per minute (dpm).

Determination of hydroxyproline and protein contents. The amount of hydroxyproline was assayed by the method of *Kivirikko et al.* (1967) and α-amino nitrogen according to *Rubinstein* and *Pryce* (1959) after the samples had been hydrolysed in 6 M HCl at 136°C for 6 hours. The collagen content was calculated by multiplying the values of hydroxyproline by a factor of 7.4 (*Neuman & Logan*, 1950 b) and the protein content was calculated by multiplying the values of α-amino nitrogen by a factor 6.8 (*Rubinstein & Pryce*, 1959).

Presentation of the values. The values for ^{14}C-hydroxyproline formation were related to the protein content of the tissue, and the activity of PPH was expressed as dpm ^{14}C-hydroxyproline synthesized in an aliquot of 50,000 dpm ^{14}C-proline-labelled protocollagen substrate per mg 15,000 $\times$ g supernatant protein. The results are presented as means $\pm$S.D.; the statistical significance of differences was tested by Student's t-test.

RESULTS

Collagen and total protein contents of pulps. Table I shows the concentrations of collagen and total protein in rabbit pulp tissue. The difference in collagen concentrations between molar and incisor pulps is statistically significant ($p<0.001$).

Synthesis of ^{14}C-hydroxyproline. The time-dependence of the formation of ^{14}C-hydroxyproline is shown in Fig. 1. The formation of ^{14}C-hydroxyproline continues linearly up to 9 hours, but thereafter the rate of synthesis decreases. The synthesis of ^{14}C-hydroxyproline paralleled the total ^{14}C-incorporation up to 9 hours, and the ratio of ^{14}C-hydroxyproline to total radioactivity $\times$ 100 varied in 6-hour experiments from 1.4 to 3.6.

Effect of pH on the synthesis of ^{14}C-hydroxyproline. As shown in Fig. 2, the maximal formation of ^{14}C-hydroxyproline takes place between the pH values 7.4 and 8.2. When using instead of HEPES buffer, 0.2 M Tris-maleate the incorporation of ^{14}C-proline was inhibited, and the radioactivity of ^{14}C-hydroxyproline in these experiments was less than one-tenth of the value obtained when HEPES buffer was used.

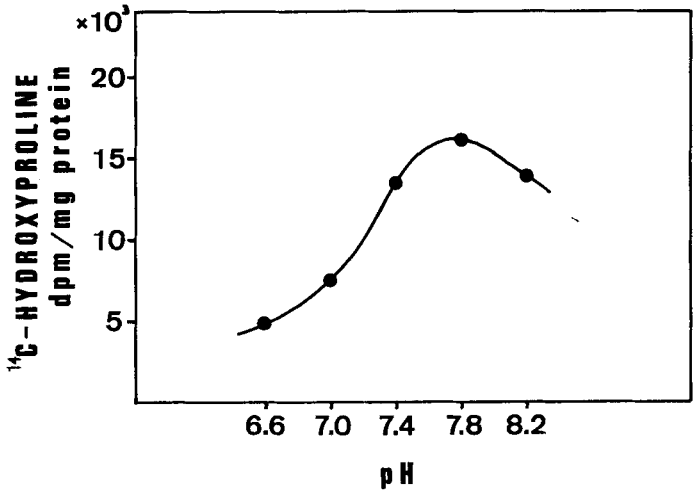

Fig. 2. Effect of pH on the formation of ^{14}C-hydroxyproline in the dental pulp in vitro. The experimental conditions same as in fig. 1. Incubation time 6 hours. The pH values those at the beginning of the experiment. Each dot represents the mean of four determinations.

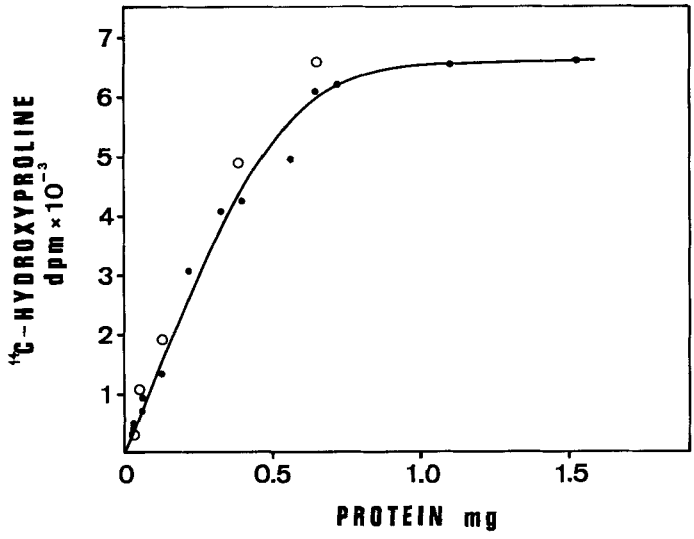

Fig. 3. Activity of protocollagen proline hydroxylase in the dental pulp. The values indicate dpm ^{14}C-hydroxyproline synthesized with 50,000 dpm ^{14}C-proline labelled protocollagen as substrate and varying amounts of 15,000×g supernatant of the rabbit pulp homogenate as the source of the enzyme. Molar pulp •; Incisor pulp O.

The protocollagen proline hydroxylase activity in rabbit pulp. Under the assay condition used the formation of ^{14}C-hydroxyproline was proportional to the amount of 15,000 × g supernatant protein up to 0.5 mg, but at higher levels deviated markedly from linearity (Fig. 3). No significant difference could be demonstrated between the enzyme activities extracted from molar and incisor pulps at the protein levels tested (Fig. 3). The quadruple freeze-thawing of intact pulps did not affect the activity of PPH.

DISCUSSION

Interest in collagen has greatly increased since it had been realized that the metabolic inactivity of collagen is only apparent, the turnover of its newly synthesized forms being, on the contrary, very rapid. With the aid of isotope techniques, many studies on the metabolism of collagen have been made both *in vivo* and *in vitro*. These studies have demonstrated changes in collagen metabolism in various clinical and experimental conditions (*Prockop & Kivirikko*, 1967). Recently, special attention has been paid to the enzyme protocollagen proline hydroxylase, which catalyzes the hydroxylation of peptide-bound proline to hydroxyproline. PPH has been demonstrated in a number of animal and human tissues and it has been shown to require molecular oxygen, ascorbate, α-ketoglutarate and ferrous ions for its activity (*Hutton & Udenfriend*, 1966; *Takeuchi et al.*, 1967; *Langner & Fuller*, 1969; *Uitto et al.*, 1969). PPH activity has been shown to be high when active synthesis of collagen takes place, e.g. in embryonic and granulomatous tissues (*Juva*, 1968; *Halme*, 1969). In addition, a parallelism has been demonstrated between the rate of collagen formation and the activity of PPH (*Juva*, 1968).

In the field of dentistry, collagen has been studied for the most part by microscopic and radioautographic methods. Biochemical information on collagen metabolism in dental tissues is mainly based on knowledge gained from studies on collagen elsewhere in the body. With radioautographic techniques, the formation of new collagen has been shown to occur all over the connective tissue of the pulp, being most abundant in the proximity of the odontoblasts on the predentine side (*Carneiro & Leblond*, 1959; *Leblond*, 1963; *Sayegh*, 1967). As shown by *Pohto* and *Antila* (1968), the cell bodies of odontoblasts are extirpated with the pulp. Therefore, the results obtained by the methods presented in this report include the formation of new collagen in both fibroblasts and odontoblasts.

In spite of being open-rooted, rabbit teeth contain pulps which are of

great value in endodontic studies and comparable to human pulps, as well (*Slavkin & Bavetta,* 1968). Moreover, the collagen of the rabbit has been shown to bear a strong resemblance to human collagen in over-all amino acid composition and chain structure (*Bornstein & Nesse,* 1970).

The ground substance of dental pulp is known to decrease and the fibrillar material to increase with age (*Zerlotti,* 1964; *Bhussry,* 1968), resulting in a state which has been described by *Amies* (1944) as »senile fibrosis». The observation of a relatively higher collagen content in molar pulps than in incisor pulps is consistent with the hypothesis that in tooth development in the rabbit the molars represent a more mature form than the incisors.

An incubation method essentially similar to that presented in this work has recently been tested for suitability in studies on collagen formation in human skin (*Uitto,* 1970). In those experiments it was observed that phosphate ion, when present in the incubation medium as part of Krebs-Ringer solution, reduces the synthesis of collagen to one-third of that occurring when phosphate ion is absent. This ion, as an integral constituent of many filling and lining materials, has been observed to be able to escape through the dentine into the pulpal tissue (*Swarts et al.,* 1968; *Johnson et al.,* 1970). In addition, as seen in vital microscopy, many material commonly used in dentistry, when applied to exposed dentine, extend their influence to the pulp, disturbing its functional balance (*Pohto & Scheinin,* 1962, 1967). According to the results reported here, the formation of collagen hydroxyproline is very sensitive to changes in the incubation medium, e.g. pH and the composition of the buffer solution used. For this reason it can well be understood that the anabolic stage in pulpal collagen metabolism is apt to be disturbed when acidic or basic material is laid on the dentine.

The methods used in this study seem to be useful tools for further investigations designed to ascertain the effects of the various procedures of endodontic and operative dentistry on the metabolism of collagen in the dental pulp.

Acknowledgement. This study has been partly supported by a grant from the National Research Council for Medical Sciences, Finland. The rabbits used in this study were kindly supplied by Medica ltd., Finland.

REFERENCES

Amies, A., 1944: Diseases of the pulp. Austr. J. Dent. *48:* 97.

Bhussry, B. R., 1968: Modification of the dental pulp organ during development and aging. In: Biology of the dental pulp organ: A Symposium, ed. S. B. Finn. p. 145. University of Alabama Press.

Bornstein, P. & R. Nesse, 1970: The comparative biochemistry of collagen: The structure of rabbit skin collagen and its relevance to immunochemical studies of collagen. Arch. Biochem. Biophys. *138:* 443.

Carneiro, J. & C. P. Leblond, 1959: Role of osteoblasts and odontoblasts in secreting the collagen of bone and dentin, as shown by radioautography in mice given tritium labelled glysine. Exp. Cell. Res. *18:* 291.

Chvapil, M. & J. Hurych, 1968: Control of collagen biosynthesis. Int. Rev. Connect. Tissue Res. *4:* 67.

Halme, J., 1969: Development of protocollagen proline hydroxylase activity in chick embryo. Biochem. Biophys. Acta *192:* 90.

Halme, J., K. I. Kivirikko & K. Simmons, 1970: Isolation and partial characterization of highly purified protocollagen proline hydroxylase. Biochim. Biophys. Acta *198:* 460.

Hutton, J. J. & S. Udenfriend, 1966: Soluble collagen proline hydroxylase and its substrates in several animal tissues. Proc. Nat. Acad. Sci *56:* 198.

Johnson, R. H., G. J. Christensen, R. W. Stigers & R. H. Laswell, 1970: Pulpal irritation due to the phosphoric acid component of silicate cement. Oral Surg. Oral Med. Oral Path. *29:* 447.

Juva, K. & D. J. Prockop, 1966: Modified procedure for the assay of H^3 or C^{14} labelled hydroxyproline. Anal. Biochem. *15:* 77.

Juva, K., 1968: Hydroxylation of proline in the biosynthesis of collagen. Acta Physiol. Scand. (Suppl.) p. 308.

Kivirikko, K. I. & D. J. Prockop, 1967: Enzymatic hydroxylation of proline and lysine in protocollagen. Proc. Nat. Acad. Sci. U.S. *57:* 782.

Kivirikko, K. I., O. Laitinen & D. J. Prockop, 1967: Modifications of a specific assay for hydroxyproline in urine. Anal. Biochem. *19:* 249.

Kivirikko, K. I., 1970: Urinary excretion of hydroxyproline in health and disease. Int. Rev. Connect. Tissue Res. *5:* 93.

Langner, R. O. & G. C. Fuller, 1969: Elevation of proline hydroxylase activity in diseased rabbit aorta. Biochem. Biophys. Res. Commun. *36:* 559.

Leblond, C. P., 1963: Elaboration of dental collagen in odontoblasts as shown by radioautography after injection of labeled glysine and proline. Ann. Hictochim. *8:* 43.

Manner, G., R. H. Kretsinger, B. S. Gould & A. Rich, 1967: The polyribosomal synthesis of collagen. Biochim. Biophys. Acta *134:* 411.

Neuman, R. E. & M. A. Logan, 1950 a: The determination of hydroxyproline. J. Biol. Chem. *184:* 299.

—»— 1950 b: The determination of collagen and elastin. J. Biol. Chem. *186:* 549.

Pohto, M. & A. Scheinin, 1962: Effect of local analgesic solutions on the circulation of the dental pulp. Int. dent. J. *12:* 227.

»— 1967: Vital microscopy of the pulp in the rat incisor VII. Reaction to silicate cements. Suom. Hammaslääk. Toim. *63:* 178.

Pohto, P. & R. Antila, 1968: Acetylcholinesterase and noradrenaline in the nerves of mammalian dental pulps. Acta odont. scand. *26:* 641.

Prockop, D. J. & P. S. Ebert, 1963: A simple method for differential assay of tritium and carbon-14 in water-soluble biological materials. Anal. Biochem. *6:* 263.

Prockop, D. J. & K. I. Kivirikko, 1967: Relationship of hydroxyproline excretion in urine to collagen metabolism. Ann. Int. Med. *66:* 1243.

Rubinstein, H. & J. D. Pryce, 1969: The colorimetric estimation of alpha-amino nitrogen in tissue fluids. J. Clin. Path. *12:* 80.

Sayegh, F. S., 1967: Healing of the traumatized dental pulp. J. Dent. Res. *46:* 1036.

Slavkin, H. C & L. A. Bavetta, 1968: Total protein, collagen, nucleic acids and calcium ions during rabbit odontogenesis. Archs. oral Biol. *13:* 1465.

Solomons, C. C., J. T. Irving & W. F. Neuman, 1960: Calcification in biological systems. Am. Assos. Advance. Sci., Washington, D.S. 203.

Swarts, M. L., P. F. Niblack, E. A. Alter, R. D. Norman & R. W. Phillips, 1968: In vivo studies on the penetration of dentine by constituents of silicate cement. J. Amer. dent. Ass. *76:* 573.

Takeuchi, T., K. I. Kivirikko & D. J. Prockop, 1967: Increased protocollagen hydroxylase activity in the livers of rats with hepatic fibrosis. Biochem. Biophys. Res. Commun. *28:* 940.

Uitto, J., 1970: A method for studying collagen biosynthesis in human skin biopsies in vitro. Biochem. Biophys. Acta *201:* 438.

Uitto, J., J. Halme, M. Hannuksela, P. Peltokallio & K. I. Kivirikko, 1969: Protocollagen proline hydroxylase activity in the skin of normal human subjects and of patients with scleroderma. Scand. J. Clin. Lab. Invest. *23:* 241.

Van Slyke, D. D., A. Hiller & D. A. MacFadyen, 1941: The determination of hydroxylysine in proteins. J. Biol. Chem. *141:* 681.

Weinstein, E., N. Blumenkrantz & D. J. Prockop, 1969: Hydroxylation of proline and lysine involves two separate enzymatic sites. Biochim. Biophys. Acta *191:* 747.

Veis, A., 1964: The macromolecular chemistry of gelatin. Academic Pres, Inc., Publishing, New York.

Zerlotti, E., 1964: Histochemical study of connective tissue of the dental pulp. Archs. oral Biol. *9:* 149.

Address:
Veli-Jukka Uitto,
Department of Medical Chemistry,
University of Helsinki,
Siltavuorenpenger 10,
Helsinki 17, Finland