Collagen Metabolism in Rat Incisor Predentine in Vivo: Synthesis and Maturation of Type I, α_1 (I) Trimer, and Type V Collagens†

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ABSTRACT: Collagen synthesis and deposition in the predentine of the continuously erupting rat incisor was analyzed in vivo following a single intraperitoneal injection of [^{14}C]glycine. Newly synthesized collagen was extracted from the dissected predentine with a solution of 1.0 M sodium chloride containing proteolytic enzyme inhibitors. Mature, cross-linked collagen was solubilized by limited pepsin digestion following extractions with 0.5 M acetic acid or 0.1 M penicillamine. Analysis of the radiolabeled collagens by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography showed that over 97% of collagen synthesized migrated in the α_1 (I) and α_2 positions following pepsin digestion; the remaining 3% migrated in the positions of α_1 (V) and α_3 (V) collagens. However, from the ratio of the α_1 (I):α_2 it was estimated that approximately 30% of the salt-extractable collagen was α_1 (I) trimer. The presence of this collagen was confirmed by salt-fractionation and cyanogen bromide digestion patterns. Type I, α_1 (I) trimer, and type V collagens were also found in the salt-insoluble tissue residue. In this fraction the α_1 (I) trimer comprised 10–15% of the collagen measured as radioactivity but was difficult to discern colorimetrically. Type III collagen could not be detected in any of the fractions analyzed. From the profiles of isotope incorporation into collagens and collagen precursors, it was evident that collagen synthesis and processing was rapid. Processing of type I collagen and probably also α_1 (I) trimer proceeded almost entirely through procollagen intermediates. Rapid maturation of the types I and V collagens was evident from the sharp increase in radiolabeled collagen in the salt-insoluble fraction and the appearance of β and γ chains as early as 30 min after isotope administration. Radiolabeled procollagens were also extracted with acetic acid and penicillamine, indicating that cross-linking of collagen precursors may be involved in fiber formation.

The continuous formation of dentine in the rodent incisor provides a unique system to study the formation of a mineralized connective tissue. In the process of dentinogenesis, odontoblasts produce a collagenous matrix, predentine,[1] which subsequently becomes mineralized to form dentine. Previous studies have shown that type I collagen is the major collagen present in rat (Butler & DeSteno, 1971) and bovine (Scott & Veis, 1976) dentine and also in bovine predentine (Dodd & Carmichael, 1979). Some biochemical (Volpin & Veis, 1973) and histochemical (Cournil & Pomponio, 1977) evidence has been presented to suggest the presence of type III collagen in dentine. Type III collagen has also been observed in predentine but not dentine in mouse tooth germ (Thesleff et al., 1979). However, studies by Scott & Veis (1976) have failed to demonstrate type III collagen in dentine. Studies on rat odontoblasts in culture (Munksgaard et al., 1978) and on collagen extracted from the dentine of lathyritic rats (Wohllebe & Carmichael, 1978) have respectively demonstrated the synthesis and deposition of α_1 (I) trimer [α_1 (I)$_3$][2] in this tissue. However, the significance of these findings with respect to normal tissue in vivo has not been established. Indeed in a recent study Munskgaard & Moe (1980) found less than 3% α_1 (I) trimer in neutral-salt and dilute acetic acid extracts of dentine in developing bovine teeth.

The combination of NaDodSO$_4$[3]–polyacrylamide gel electrophoresis and fluorography provides an extremely sensitive technique which can be used to demonstrate the synthesis of different collagens radiolabeled in vivo (Limeback & Sodek, 1979; Sodek & Limeback, 1979). Further, in tissues with a high rate of collagen synthesis, it is possible to study procollagen conversion and collagen maturation. We have used this technique to determine the nature of collagens synthesized by the odontoblasts in predentine and to demonstrate the mode of procollagen conversion and collagen maturation of the major collagen types in this tissue.

Materials and Methods

In Vivo Labeling. Male and female CBL Wistar rats weighing 220 ± 10 g were given 1.5 mCi of [^{14}C]glycine (NEC-047H; New England Nuclear) by intraperitoneal injection. Rats were killed starting 0.5 h after administration of isotope; the latest time point studied was at 6 h. The mandibular incisors were quickly dissected out, and under a dissecting microscope, the teeth were split longitudinally. The pulp was carefully removed and the predentine scraped from the surface of the pulp cavity and placed into a 1.5-mL microcentrifuge tube containing 1.0 mL of 1.0 M NaCl in 0.05 M Tris-HCl buffer, pH 6.8, at 4 °C. The following proteolytic enzyme inhibitors were also present in the extracting solution: (ethylenedinitrilo)tetraacetic acid, 10 mM; *N*-ethylmaleimide, 10 mM; phenylmethanesulfonyl fluoride, 100 μM; leupeptin, 10 μg/mL; pepstatin, 1 μg/mL. The predentine samples were extracted for 2 h at 4 °C on a rotary platform operating at 200 rpm. Two successive extractions in fresh extracting solution were carried out over the next 20 h. Between extractions the tissue was sedimented by centrifugation at 10000*g* in a microfuge (Brinkman) for 5 min. The tissue was washed in 1.0 mL of cold distilled water which was combined with the preceding extracts. Tissue residues were then extracted with either 2 × 1.0 mL of 0.5 M acetic acid containing leupeptin and pepstatin at the same concentration as in the first ex-

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[1] In this study, predentine refers to the tissue synthesized by odontoblasts and includes the odontoblasts themselves.

[2] We have used the symbol α_1 (I)$_3$ or the term α_1 (I) trimer to describe that form of collagen believed to comprise three α_1 chains that are similar to or identical with the α_1 chains of type I collagen.

[3] Abbreviations: NaDodSO$_4$, sodium dodecyl sulfate; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; CNBr, cyanogen bromide; pc, procollagen intermediate with the amino-terminal propeptide removed.

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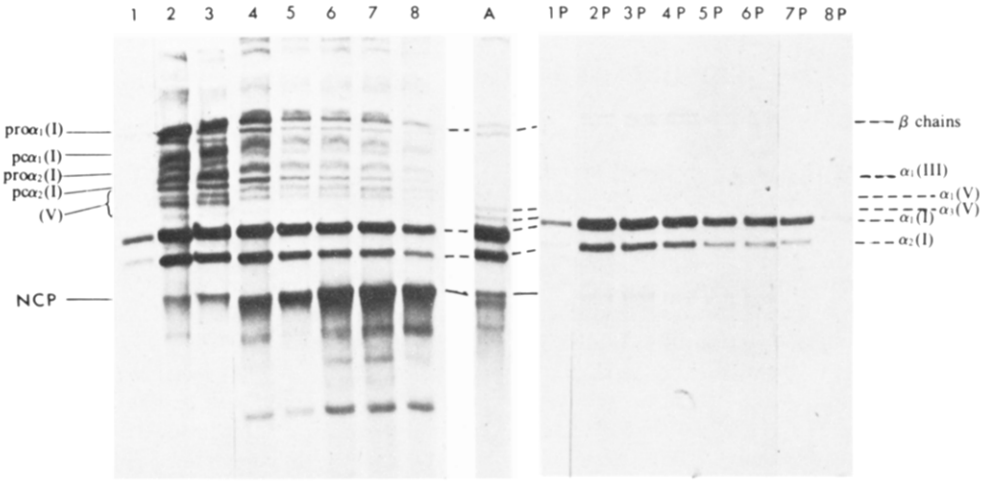

FIGURE 1: Fluorographs of radiolabeled collagens and procollagens extracted from predentine with 1.0 M NaCl and separated by $NaDodSO_4$–polyacrylamide gel electrophoresis using delayed reduction before (1–8) and after (1P–8P) pepsin digestion. Tracks 1–8 correspond to samples collected 0.5, 0.7, 0.9, 1.25, 2, 3, 4, and 6 h after isotope administration. Track A is a longer exposure of a pepsin-digested sample to more clearly demonstrate the β chains, the type V collagens, and the pepsin-resistant NCP.

tracting solution or with 2 × 1.0 mL of 1.0 M sodium chloride, pH 7.0, containing 0.1 M penicillamine and the full complement of proteolytic enzyme inhibitors. The tissue residue remaining after acetic acid extraction was washed with inhibitor-free 0.5 M acetic acid and then digested with 100 μg of pepsin (Worthington 3× crystallized) in 1.0 mL of 0.5 M acetic acid at 22 °C for 24 h. After a second 24-h digestion with 50 μg of pepsin, the solubilized material was pooled.

The salt, penicillamine, and acetic acid extracts were centrifuged at 100000*g* in an IEC ultracentrifuge and the salt and penicillamine supernatants dialyzed against distilled water. After samples were taken to determine radioactivity, aliquots were freeze-dried for subsequent analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis. Pepsin digests of salt-extractable samples were carried out at 15 °C for 4 h with 100 μg of pepsin in 0.5 mL of 0.5 M acetic acid.

Analysis by $NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Electrophoresis in $NaDodSO_4$ was carried out on 20-cm slab gels as described previously (Sodek & Limeback, 1979). Gels were either stained with Coomassie brilliant blue or prepared for fluorography. Stained gels were also subjected to fluorography after removing most of the stain by prolonging the washes in Me_2SO. Fluorographs were scanned by using a Gilford spectrophotometer with appropriate attachments, and quantitation was achieved by measuring the peaks with an integrating digital densitometer (Hommel Electronics Toronto). Identification of type I procollagens and type V collagen was based on comparative migration of known standards as well as their susceptibility to bacterial collagenase.

Identification of α_1 (I) Trimer. The relative amounts of radiolabeled α_1 (I) and α_2 chains were determined from fluorographs by scanning densitometry of salt-extracted and pepsin-solubilized mature collagens of predentine and several periodontal tissues. Standard curves were constructed relating the proportion of α_2 chains to the total radioactivity (measured in arbitrary units). The ratio of α_1 (I) to α_2 for predentine was obtained by extrapolation from curves of those samples believed to contain little or no α_1 (I) trimer.

For confirmation of the presence of α_1 (I) trimer in the newly synthesized pool, salt-extracted radiolabeled material from animals killed 0.75, 1.25, 1.40, and 2.5 h after administration of isotope was dialyzed to equilibrium against 2.5 M sodium chloride at neutral pH in the presence of 0.3–0.5 mg/mL type I collagen carrier. The precipitated material was pelleted by centrifugation at 10000*g* for 10 min and washed once with 0.5 mL of 2.5 M sodium chloride solution. Samples of the pellets and supernatants were dialyzed against water and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography. A similar procedure was used for the analysis of the pepsin-solubilized insoluble collagens except that carrier collagen was not added.

Radiolabeled material in the 2.5 M supernatants and radiolabeled α_1 (I) standard prepared from monkey collagen by CMC chromatography were freeze-dried and dissolved in 1.0 mL of 70% (v/v) formic acid in a test tube. Solid CNBr was added to a concentration of approximately 0.5% (w/v) and the tube flushed out with nitrogen. After being incubated for 4 h at 22 °C, the digest was diluted 5-fold with water and freeze-dried. The CNBr peptides were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis using a 10% separating gel.

Results

Salt-Extracted Material. Radiolabeled proteins extracted with 1.0 M NaCl from rat predentine over the 6-h period studied are shown in the fluorograph of samples analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1). At the early time points radiolabeled bands corresponding to pro-α_1 (I) and pro-α_2 could be clearly resolved together with their corresponding pc intermediates. The intensity of the bands decreased rapidly after 0.9 h whereas the α_1 and α_2 chains, which were already prominent at the 0.5-h point, increased to a maximum at 1.25 h and also declined rapidly thereafter. A series of collagenase-digestible radiolabeled bands, which migrated faster than the pc α_2 and were unaffected by mercaptoethanol treatment, appear to represent the naturally processed forms of type V collagens.

Following pepsin digestion the procollagens in each sample were converted to α chains. Analysis of the products (Figure 1) showed radiolabeled bands corresponding to α_1 (I) and α_2 chains and β chains which were evident from the 0.5-h time point. In addition, two weaker bands with the migratory characteristics of the α_1 and α_3 chains of type V collagen were observed. However, there was no indication of any radiolabeled material migrating in the position expected for type III collagen α chains. Quantitative analysis of the radioactivity associated with the α_1 and α_2 chains and the corresponding precursors indicated a greater ratio than the 2:1 ratio (actual values varied from 3:1 to 5:1) expected for type I collagen. A series of diffuse bands (approximately 70000 daltons)

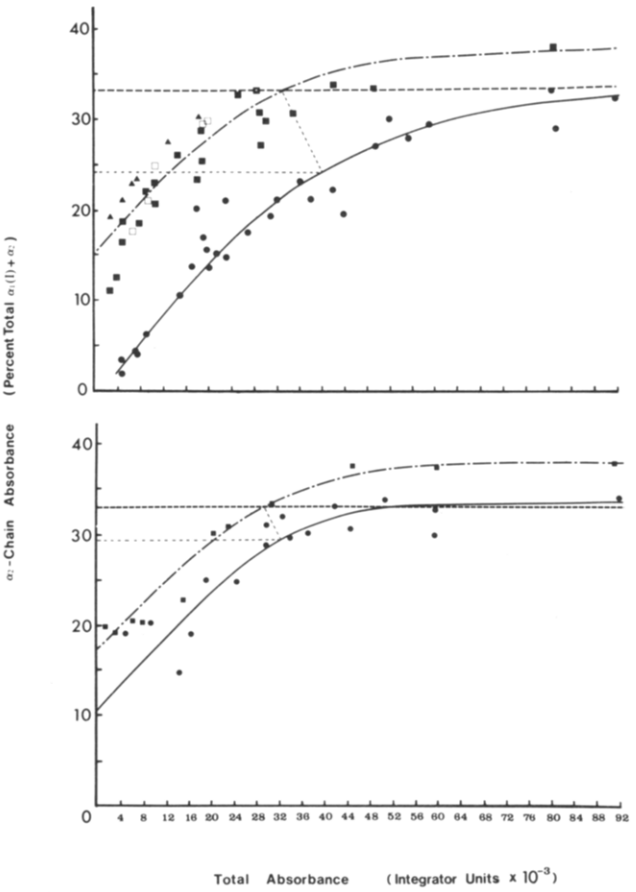

FIGURE 2: Quantitation of α_1:α_2 ratios from fluorographs of samples of salt-extractable (upper) and pepsin-digested salt-inextractable (lower) collagens. Since the fluorographic response and radioactivity do not demonstrate a linear relationship, ratios were calculated for various samples at various fluorographic exposures and expressed against the total absorbance. Several periodontal tissues [(▲) gingiva; (■) incisor ligament; (□) molar ligament] in which the α_1:α_2 ratio is expected to be 2:1 were compared to predentine (●) samples. The excess α_1 chains in the predentine samples was calculated by drawing a line at right angles to a tangent of the curve for periodontal tissues where the curve is intersected by the line (---) which indicates the theoretical value for a 2:1 ratio (i.e., $33^1/_3$% α_2). The α_2-chain percentage for the predentine samples was obtained by extrapolating from the point of intersection of the drawn line on the predentine curve as shown.

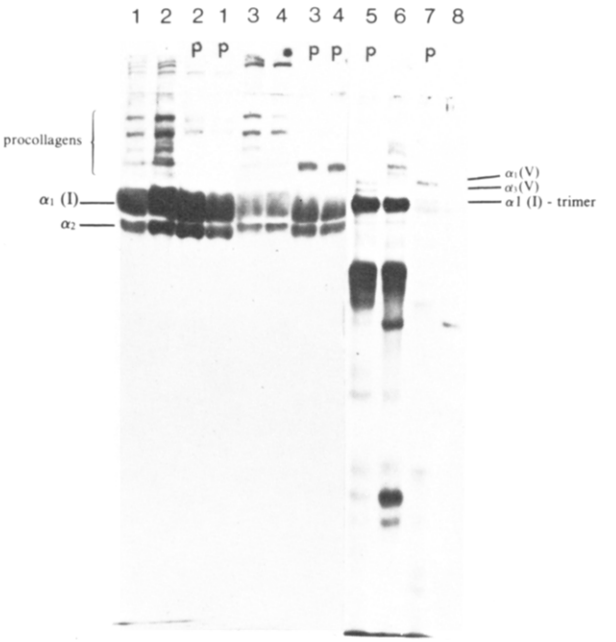

FIGURE 3: Identification of α_1 (I) trimer in the salt-extractable pool was initiated by adding 0.3–0.5 mg of type I carrier collagen to radiolabeled extracts of predentine and incisor ligament and dialyzing against 2.5 M NaCl at neutral pH. Radiolabeled material in the precipitates that formed and the supernatants was analyzed by Na-DodSO_4–polyacrylamide gel electrophoresis using delayed reduction. The first eight tracks show the material in the precipitates of samples of 2.5- (1, predentine; 4, incisor ligament) and 0.75-h (2, predentine; 3, incisor ligament) time points. Equivalent samples digested with pepsin are designated P. The last four tracks show the material in the supernatants of predentine (5) and ligament (7) after pepsin digestion and (6) predentine and (8) ligament before pepsin digestion of the 0.75-h samples.

migrating faster than the α_2 chains were also observed in the pepsin-digested samples. From their migration position, their resistance to collagenase, and the increase in intensity of these bands with time, they would appear to be derived from the noncollagenous proteins labeled NCP in Figure 1.

Salt-Inextractable Material. Extraction of the tissue residue remaining after neutral salt extraction with either 0.5 M acetic acid or 0.1 M penicillamine solubilized small but significant amounts of radiolabeled material with the characteristics of procollagens and collagen α chains. Following pepsin digestion, the procollagen bands were lost, and the α-chain bands were increased. The radioactivity associated with the procollagens was prominent at early times but decreased rapidly thereafter (supplementary material).

Pepsin digestion of tissue residues extracted with acetic acid or penicillamine released collagen α chains corresponding to types I and V collagens. No evidence of type III collagen was obtained. Fluorographic analysis of these samples showed a progressive increase in the radioactivity associated with the type I collagen α chains, the α_1 (V) and α_3 (V) chains, and the β chains. Analysis of the specific radioactivities (radioactivity related to the corresponding colorimetric amount), however, demonstrated lower values for the type V collagen chains.

Analysis of α_1 (I) Trimer. The ratio of α_1 (I):α_2 for radiolabeled collagens from predentine and several periodontal tissues, analyzed at varying concentrations and with different fluorographic exposure times, is shown in Figure 2. In salt-extracted samples of predentine, the α_1 (I):α_2 ratio was consistently higher than those of all the other samples when the α_1 (I) chain was not over exposed (below 40 000 units). Under conditions where the α_1 (I):α_2 ratio was 2:1 for most periodontal tissues, a ratio of 3.4:1 was obtained for predentine samples. A similar ratio was obtained with samples analyzed after pepsin digestion (results not shown). By use of the same approach for the α chains obtained from pepsin digestions of the salt-inextractable tissue residue (Figure 2), the α_1 (I):α_2 ratio was calculated as 2.5:1 for the radiolabeled material and 2.0:1 colorimetrically.

For determination of the nature of the excess radiolabeled material migrating in the α_1 (I) position, samples of salt-extractable and pepsin-digested, salt-inextractable material were subjected to fractionation with 2.5 M sodium chloride. Analysis of precipitates formed and supernatants is shown in Figure 3. Both procollagens and α chains of type I and III collagens from predentine and incisor ligament in salt-extractable material were precipitated. Subsequent digestion with pepsin converted procollagens to α chains. In the supernatants of predentine, a prominent band was observed in the α_1 (I) position with minor bands in the region of procollagens and a band corresponding to the NCP. Pepsin digestion resulted in the loss of "procollagen" bands. In addition, bands corresponding to type V α_1 and α_3 chains were apparent following pepsin digestion, suggesting that some of the original

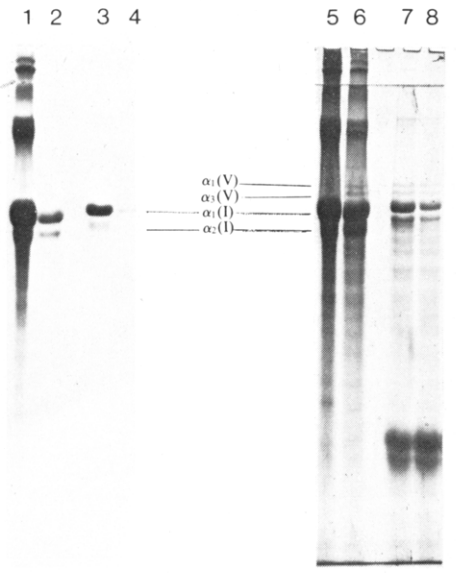

FIGURE 4: Identification of α_1 (I) trimer in the salt-inextractable pool of predentine was made by dialyzing pepsin-solubilized material against 2.5 M NaCl at neutral pH. The precipitates (tracks 1, 2, 5, and 6) and supernatants (tracks 3, 4, 7, and 8) were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis, stained with Coomassie-blue (5–8), and then treated for fluorography (1–4). Tracks 1, 3, 5, and 7 correspond to tissue samples taken at 2.5 h and tracks 2, 4, 6, and 8 at 0.75 h following isotope administration.

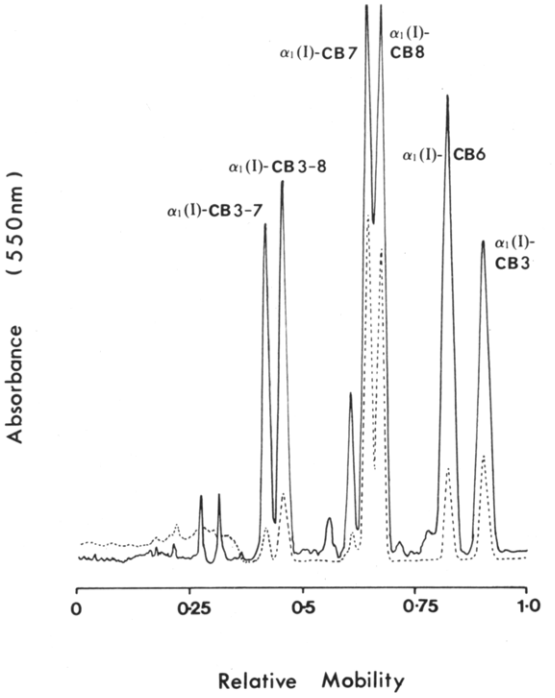

FIGURE 5: Fluorograph tracings of CNBr peptides obtained from radiolabeled monkey α_1 (I) purified on CM-cellulose (solid line) and the 2.5 M NaCl supernatant from the salt extracts of predentine shown in Figure 3 (broken line).

bands corresponded to naturally processed forms of type V collagen.

Most of the material released from the salt-inextractable tissue residue by pepsin digestion was recovered in the 2.5 M sodium chloride precipitate as α_1 and α_2 chains in a ratio of close to 2:1 (Figure 4). However, an excess of radiolabeled α_1 (I) chains appeared in the supernatants. Although material was also apparent below the α_1 chains, this material was noted to migrate slower than the α_2 band, and the specific radioactivity of this material was much lower than the α_1 (I). Also it was observed that in unfractionated material a double peak was observed in the α_2-chain position.

For identification of the nature of the collagen migrating in the α_1 (I) position, CNBr fragments of the radiolabeled material from the 2.5 M sodium chloride supernatant were prepared and shown to be essentially identical with those obtained from authentic α_1 (I) chains (Figure 5).

Discussion

Using $NaDodSO_4$–polyacrylamide gel electrophoresis to separate radiolabeled collagens from rat incisor predentine, this study has demonstrated that 97% of the collagen synthesized and deposited into the tissue matrix of predentine comprises α_1 (I) and α_2 chains. In contrast to the results of Volpin & Veis (1973), Cournil & Pomponio (1977), and Thesleff et al. (1979), type III collagen was not observed. The remaining 2–3% of collagenous material was identified as α_1 (V) and α_3 (V) collagens based on mobilities, degradation by bacterial but not by vertebrate collagenases, and solubility in 2.5 M NaCl. From the α_1 (I):α_2 ratio it was evident that as much as 30% of the collagen synthesized comprises α_1 (I) trimer, the identity of this collagen being confirmed by CNBr patterns of 2.5 M NaCl soluble material. However, α_1 (I) trimer represented only 10–15% of the radiolabeled, mature collagen and was difficult to demonstrate in colorimetric analyses. Munskgaard et al. (1978) found a 5–6:1 ratio for α_1 and α_2 chains synthesized by rat incisor odontoblasts in vitro, and a 3:1 ratio was found by Wohllebe & Carmichael (1978) in extracts of lathyritic rat dentine. In view of the observations of Wohllebe & Carmichael (1978) and our results with the salt-extractable collagens, it is unlikely that the discrepancy between the amounts of α_1 (I) trimer in the two pools is due to selective degradation of the α_1 (I) trimer by pepsin. Since the amount of α_1 (I) trimer does not appear to increase in the salt-extractable pool with time, it is possible that much of it is degraded before and after cross-linking. However, since approximately 10% of the radioactivity and hydroxyproline was not solubilized by pepsin digestion of the salt-inextractable tissue, a covalent association of the α_1 (I) trimer with a pepsin-resistant macromolecule could also explain these observations.

Our demonstration of the synthesis of significant amounts of α_1 (I) trimer is the first reported in vivo. Although this form of collagen has been demonstrated in several tissue culture systems (Mayne et al., 1975, 1976a,b, 1978; Benya et al., 1977; Narayanan & Page, 1976; Daniel, 1976; Muller et al., 1974; Little et al., 1977; Schiltz et al., 1977; Munksgaard et al., 1978; Hata & Slavkin, 1978; Crouch & Bornstein, 1978) and has been extracted from diseased tissues (Moro & Smith, 1977) and lathyritic tissues (Jiminez et al., 1977; Wohllebe & Carmichael, 1978), these observations could result from aberrations in the normal synthetic pattern of connective tissues through culture conditions, pathological influences, and responses to drug administration. Although small amounts of α_1 (I) trimer have been extracted from skin (Uitto, 1979), synthesis of large amounts of α_1 (I) trimer appears to be a normal physiological event in the activity of rat odontoblasts. The significance of the production of this collagen and its apparent transient existence in the formation of predentine is not readily evident, particularly as little α_1 (I) trimer has been found in salt and acid extracts of bovine predentine (Munksgaard & Moe, 1980).

The rapid loss of radiolabeled procollagen precursors in the salt-extractable pool with time and the sharp increase in α chain radioactivity over the first 1.25 h is indicative of a rapid

processing of procollagen molecules. The subsequent decline in radiolabeled pc intermediates demonstrates the stepwise processing of type 1 procollagens beginning with the removal of the amino-terminal propeptide (pn peptide). This form of procollagen processing has been found to be the predominant pathway for most tissues in vitro (Bornstein & Sage, 1980) and for rat periodontal tissues (Sodek & Limeback, 1979) and uterus (S. M. Mandell and J. Sodek, unpublished results) in vivo.

Since procollagen precursors of the α_1 (I) trimer in fetal membranes have been shown to be similar to the α_1 (I) precursors (Crouch & Bornstein, 1979), rapid processing via a pc intermediate is probable for the α_1 (I) trimer in predentine. This is supported by the observation in these studies that the ratios of the pro-α_1:pro-α_2 and pc α_1:pc α_2 bands are greater than 2:1. Rapid processing of the precursors of the α_1 (V), α_3 (V), and possibly also the α_2 (V), which comigrates with the α_1 (I) chain, is also indicated by the observation of three collagenase digestible bands, unaffected by mercaptoethanol treatment, which migrated in positions similar to those shown by Kumamoto & Fessler (1980) for the processed forms of α_1 (V) and α_2 (V) in chick crop in vitro. The identification of these bands is supported in our study by their solubility in 2.5 M NaCl.

Although procollagen processing is rapid in predentine, the extraction of unprocessed procollagen with acetic acid and penicillamine suggests that some procollagen is stabilized, possibly through covalent cross-linking. Since the acid- and penicillamine-labile aldimine cross-link is a minor cross-link in dentine, it is conceivable that much more radiolabeled procollagen may be present in the mature collagen pool. Whether or not this material is subsequently processed cannot be determined from our study, but the immunochemical studies of Cournil et al. (1979) have shown predentine to give a strong reaction to type I procollagen antibodies. Radiolabeled procollagens have also been extracted by acetic acid and penicillamine in larger amounts in rat incisor pulp and rat uterus (S. M. Mandell and J. Sodek, unpublished results).

The rapid decline in radiolabeled collagen in the salt-soluble pool after 1.25 h together with the rapid increase in the specific radioactivity of the insoluble collagen demonstrates that the newly synthesized collagen is rapidly cross-linked and presumably deposited as fibers in the predentine matrix. Notably rapid cross-linking occurs for the type V collagen as well as type I collagen, but it is not known whether the type V collagens form fibers in the predentine matrix. Rapid cross-linking is also evident from the appearance of β chains in the salt-extractable pool as early as 0.5 h, and from β and γ chains in the insoluble fraction.

The pepsin-resistant radiolabeled protein found in the salt extracts appears to be unique to dentine since we have found only small amounts in radiolabeled pulp tissue and none at all in many other soft connective tissues we have analyzed. The material has a molecular weight of approximately 70 000 which suggests that it may be a phosphoprotein (Dimuzio & Veis, 1978) which would be labeled quite well with [^{14}C]glycine.

Acknowledgments

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Supplementary Material Available

Profiles of radioactivity in procollagens and collagens in salt-extractable and salt-inextractable pools together with fluorographs showing the progressive incorporation of radiolabeled collagens into the mature collagen pool, and the occurrence of procollagens in acetic acid extracts (1 page). Ordering information is given on any current masthead page.

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