Non-collagenous proteins of predentin from dentinogenically active bovine teeth

Mats JONTELL and Anders LINDE*

Laboratory of Oral Biology, Department of Histology, University of Göteborg, Göteborg, Sweden

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Predentin(e) was dissected out from unerupted permanent bovine teeth. The non-collagenous proteins were extracted at -13°C by 4 M-guanidinium chloride containing proteinase inhibitors and separated by DEAE-Sephadex and Sephadex G-100 chromatography. In addition to a few minor constituents, the only major non-collagenous components that could be demonstrated were albumin and proteoglycan. The localization of the former, demonstrated by optical-microscopical immunocytochemistry, was such that it was concluded that albumin is not a constituent of predentin matrix. Very low amounts of phosphoprotein were found in predentin matrix. This was of two types, high- and low-phosphorylated. Larger amounts of phosphoprotein were not present until the dissection was carried deeper into newly formed dentin(e). On the basis of the present results and previously obtained morphological data the conclusion was drawn that predentin matrix, containing virtually only collagen type I and proteoglycan, is similar in composition to that of loose connective tissue and primarily aimed at the production and maturation of collagen fibres. Only immediately before the mineralization front are the non-collagenous protein components secreted that initiate and govern calcium-phosphate mineral formation.

The same sequence of events seems to take place in dentin(e) formation and the appositional formation of bone. From a morphological point of view the calcification process occurs in two phases; the hard-tissue-forming cells produce a collagenous matrix, predentin(e) and osteoid respectively, and this is subsequently mineralized at some distance away from the cells, i.e. after a certain time lag. The detailed mechanisms involved are not fully understood, but it is generally agreed that the matrix proteins are important for the formation of calcium phosphate mineral at the mineralization front (Linde, 1982). A simplistic view, often encountered in the literature, and actually also reflected in the names 'predentin' and 'prebone' (osteoid), is that these structures consist simply of dentin and bone matrix without the mineral component.

The major component of bone and dentin matrix is collagen type I. However, owing to their physico-

chemical properties, non-collagenous proteins (NCPs) have recently attracted more attention as potentially being of major importance in the calcification process. The NCPs of dentin and bone fall into four categories: acidic glycoproteins, γ -carboxyglutamate-containing proteins, phosphoproteins and proteoglycans (Linde *et al.*, 1980, 1983). In addition, serum proteins have been isolated from these tissues (Ashton *et al.*, 1976; Butler *et al.*, 1981).

Pure osteoid is virtually impossible to obtain. Predentin, on the other hand, may be dissected out and is only slightly contaminated with its own source, odontoblasts (Linde, 1973). Collagen in predentin appears to be similar in composition to that in dentin (Carmichael *et al.*, 1975). A few biochemical studies on proteoglycan components in predentin have been published (Engfeldt & Hjerpe, 1972; Linde, 1973; Hjerpe & Engfeldt, 1976), and these macromolecules have also been studied by histological techniques (Nygren *et al.*, 1976; Sundström, 1971).

Little is known about the possible presence in predentin of phosphoprotein, the major NCP in dentin. The paper by Carmichael *et al.* (1975), frequently cited as showing the absence of phospho-

Abbreviations used: NCP(s), non-collagenous protein(s); FITC, fluorescein isothiocyanate; GdmCl, guanidinium chloride; SDS, sodium dodecyl sulphate; IgG, immunoglobulin G.

* To whom correspondence and requests for reprints should be sent.

protein in predentin, does in fact not show this. It gives, though, evidence that no collagen-bound phosphoprotein exists in predentin, but this is not surprising in view of recent findings that cast doubt on the existence of a dentin phosphoprotein fraction covalently linked to collagen (Linde *et al.*, 1981).

An autoradiographic study by Weinstock & Leblond (1973) demonstrated that, in contrast with collagen precursors, [³²P]phosphate-labelled material 'crosses predentin' rapidly from the odontoblasts to the dentin side of the predentin/dentin junction. In addition, those authors also demonstrated in the electron microscope the appearance at the mineralization front of a non-collagenous component believed to represent phosphoprotein.

The present study describes the analysis of NCPs of predentin dissected out from bovine unerupted teeth. To avoid 'artefactual' degradation of proteins, precautionary measures have been taken, such as the inclusion of proteinase inhibitors in extraction solutions. The present study shows that very little, if any, phosphoprotein is present in predentin. It is concluded that the predentin may be viewed as a connective-tissue matrix consisting of collagen and proteoglycan to which is added diverse NCPs at the mineralization front, thereby presumably initiating and promoting mineral crystal formation.

Experimental procedures

Materials

Mandibulae from 800 calves, in the stage of early mixed dentition, were obtained from a local slaughterhouse. The jaws were frozen within 30 min after the animal was killed. In one experiment the teeth were freshly dissected out from the jaws and predentin immediately isolated. DEAE-Sephadex CL-6B, Sephadex G-100 (superfine grade) and Sephadex G-25 medium were from Pharmacia Fine Chemicals, Uppsala, Sweden. Rabbit IgG against bovine serum and FITC-conjugated goat antiserum against rabbit IgG was bought from Dako A/S, Copenhagen, Denmark. Anti-(bovine albumin), raised in rabbit, was a gift from Dr. H. Nygren, of this Department. All other chemicals used were of the highest purity available.

Dissection and extraction

The mandibulae were thawed overnight at 4°C before use. From the jaws, permanent incisors and canines were dissected out by means of a bone cutter. These dentinogenically active teeth had a developed crown, and the root did not exceed one-third of the total tooth length. After removing the dental sacs the teeth were opened with a cutting nipper and the pulps were carefully removed in one piece. The odontoblast-predentin region was scraped out with a dental excavator. The tissue was

immediately transferred to a test tube containing 5 ml of 4 M-GdmCl containing seven proteinase inhibitors (Linde *et al.*, 1980; Termine *et al.*, 1980). The material dissected out was extracted for 3 days with daily changes of 5 ml of the GdmCl solution at -13°C. The three extracts were combined and centrifuged at 10000g for 15 min in the cold to remove the insoluble collagenous matrix. Desalting of the extract was performed on a column (2.6 cm × 50 cm) of Sephadex G-25 in distilled water. The material was freeze-dried and is hereafter referred to as 'predentin extract'. The residual insoluble collagen matrix was extensively washed with water and freeze-dried.

After predentin dissection, the whole teeth were extracted at 4°C for 7 days with several changes of 4 M-GdmCl containing proteinase inhibitors in order to extract any residual NCPs in predentin possibly still adhering to the dentin. After washing with distilled water the dentin on the pulpal side was firmly scraped with a sharp excavator to harvest newly formed dentin. The dentin material was demineralized and extracted for 3 days at -13°C by three 5 ml portions of 0.25 M-EDTA containing the same proteinase inhibitors as above (Linde *et al.*, 1980). The extract was desalted on Sephadex G-25 and freeze-dried as described above. This material is hereafter referred to as 'dentin extract'.

Examination of the purity of the predentin material

Predentin material was dissected out as described above and rapidly frozen in isopentane chilled with liquid N₂. Freeze sections were obtained in a cryostat (System Dittes-Duspiva) and these were stained with Methylene Blue and examined by optical microscopy. To further estimate tissue identity the calcium content in the predentin and dentin extracts was measured in an Eppendorf flame photometer.

Chromatography

The materials were separately chromatographed in the cold on a column (2.6 cm × 20 cm) of DEAE-Sephadex equilibrated with 20 mM-Tris/HCl buffer, pH 8.0, at 4°C. Elution was performed with a linear gradient of 0–0.5 M-NaCl over a total volume of 2000 ml. To elute the proteoglycan fraction the elution was generally stopped at about 0.4 M-NaCl and the column flushed with 1 M-NaCl in Tris buffer. The effluent was continuously monitored at 225 nm. Column fractions were, after freeze-drying, desalted on a column (2.6 cm × 50 cm) of Sephadex G-25 in distilled water and freeze-dried.

Sephadex G-100 (superfine grade) chromatography was performed at 4°C on a 1.6 cm × 97 cm column equilibrated with 0.5 M-NaCl in 20 mM-Tris/HCl buffer, pH 8.0, at 4°C. The elution rate was 5 ml/h and the effluent was monitored at

225 nm. Column fractions were desalted on a small column (0.9 cm $\times$ 15 cm) of Sephadex G-25 in distilled water and freeze-dried.

Electrophoresis

SDS/polyacrylamide-gel electrophoresis was performed in 15% (w/v) gels using the discontinuous Tris/glycine buffer system described by Laemmli (1970). The gels were stained with Coomassie Brilliant Blue (Swank & Munkres, 1971).

Double diffusion

Fraction B from DEAE-Sepharose chromatography, as well as Sephadex G-100 fractions, were dissolved in phosphate-buffered saline to a final concentration of 0.5 mg/ml. The samples were diluted twice, five times, and subjected to double diffusion in agar gel against undiluted rabbit anti-(bovine albumin) serum or rabbit anti-(bovine serum) IgG by the method of Ouchterlony (Wadsworth, 1962).

Indirect immunofluorescence

Predentin material was dissected out and immediately frozen in isopentane as described above. The tissue was sectioned in a cryostat and the presence of albumin in the tissue was studied by means of immunohistochemical technique at the optical-microscopic level. The rabbit anti-(bovine albumin) serum was diluted 1:10 with phosphate-buffered saline, and FITC-conjugated goat anti-(rabbit IgG) serum was diluted 1:20. A control experiment with only FITC-conjugated goat anti-(rabbit IgG) serum was performed.

Phosphate analysis

Samples were hydrolysed in 0.2 M-NaOH at 100°C for 15 min. Phosphate was measured by an automated molybdate assay developed for the Technicon autoanalyser (Linde *et al.*, 1980).

Carbohydrate analysis

Uronic acid was determined by a carbazole method (Bitter & Muir, 1962), with glucuronolactone as standard. Hexosamine was assayed by the Elson-Morgan method as described by Antonopoulos *et al.* (1964). The ratio between glucosamine and galactosamine was determined by gas chromatography (Holm *et al.*, 1972).

Amino acid analysis

Generally, protein samples were hydrolysed in an oil bath for 24 h in sealed tubes containing 6 M-constant-boiling HCl at 108°C. Phosphoprotein-containing samples, on the other hand, were hydrolysed in open tubes contained in a desiccator in an atmosphere saturated with 6 M-HCl (Linde *et al.*, 1980) and corrected for serine and phosphoserine

degradation as described by Richardson *et al.* (1978). The amino acid analyses were performed on a JEOL 5-AH automatic amino acid analyser with a two-column system (Moore *et al.*, 1958).

Results

The predentin material dissected out was examined by optical microscopy in order to control its purity. The material was found to be predominantly composed of predentin (Fig. 1). Parts of the odontoblastema, still adhering to the predentin, were present in small amounts in some locations. No mineralized dentin could be seen. No morphological differences were detected between the fresh material and the material that had been frozen and thawed before examination. The calcium content of the predentin extract was found to be 4% of the content in the dentin extract before desalting. Since predentin is known to contain some calcium (Nicholson *et al.*, 1977), this shows that the contamination of the predentin material by dentin was negligible.

When the total predentin extract was run on 15% (w/v)-polyacrylamide/SDS gels, one Coomassie Blue-staining band, with approximately the same electrophoretic mobility as bovine serum albumin, was seen (Fig. 2). In addition, several minor, very weakly stained, components were present.

DEAE-Sepharose chromatography of the extracts of predentin material (Fig. 3a) revealed three major components. No protein-containing material could be isolated from the two small peaks appearing between peak A and peak B.

Fraction A, eluting in the void volume, showed the presence of more than one component, but contained little protein. The amino acid analysis of fraction A showed the presence of hydroxyproline and a high content of glycine and proline (Table 1), demonstrating the presence of soluble collagen in this material. The analysis gives, however, evidence also for the presence of NCP material high in aspartate and serine.

In some runs a second component of fraction A (fraction A₂) was fairly well separated from the major peak in the void volume. This consisted of very little protein, but the amounts of organic phosphate and the amino acid analyses, with very high amounts of aspartate and serine and very few residues of several amino acids (Table 1), revealed that fraction A₂ contained a phosphoprotein of the high-phosphorylated type. Fraction A₂ was found to contain 15.5% (w/w) phosphate, which means that 34% of the serine is present as phosphoserine.

Fraction B, eluting at about 0.2 M-NaCl, obviously contained more than one component (Fig. 3a). This peak was thus divided into two parts, one major (B₁) and one minor (B₂).

Fig. 1. *Microdissected material from the odontoblast/pre-dentin region*

Freeze section (3–5 μm thick) was stained with 0.5% Methylene Blue, pH 5.6, and mounted. Predentin containing transversely and longitudinally sectioned tubules can be seen. A slight contamination by adhering odontoblasts is included. Magnification × 420.

Table 1. *Amino acid analysis of fractions from DEAE-Sepharose and Sephadex G-100 chromatography of predentin and dentin extracts*

Values are means of two determinations.

Amino acid	Fraction*...	Content (residues/1000 residues)											
		Insoluble† collagen	A	A ₂	B ₁	B ₂	C	D	α	β	E	F	BSA‡
Hydroxyproline		93	41	—¶	—	—	—	68	—	—	—	—	—
Aspartic acid		53	107	375	106	139	201	70	174	100	325	193	106
Threonine		18	30	14	58	49	42	28	51	64	7	39	62
Serine		36	99	459	69	120	161	63	181	62	467	140	47
Glutamic acid		79	97	40	135	125	136	85	104	142	19	156	154
Proline		124	97	9	56	60	38	99	46	56	15	37	53
Glycine		310	214	38	47	83	115	307	62	46	67	154	32
Alanine		121	88	16	82	71	52	108	64	84	20	47	87
Cysteine		nd§	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Valine		23	33	4	60	50	39	27	48	64	5	42	66
Methionine		6	6	1	6	8	5	2	7	7	—	2	5
Isoleucine		12	18	4	27	28	27	16	28	28	7	29	25
Leucine		27	46	6	104	78	48	30	74	110	5	37	107
Tyrosine		5	13	4	34	26	15	6	23	35	—	10	36
Phenylalanine		14	20	2	48	35	16	16	30	47	—	14	51
Lysine		26	34	15	98	64	52	24	57	91	46	54	105
Histidine		6	17	10	29	25	23	5	20	27	5	22	28
Arginine		52	42	3	44	38	31	44	30	36	11	24	40
Glucosamine		—¶	Pr¶	Pr	Pr	Pr	Pr	Pr	Pr	Pr	—	Pr	—
Phosphate**		—	—	157	—	—	—	—	47	—	—	—	nd

* See the text and Figs. 3 and 5 for explanation of these designations.

† Residual collagen fraction after GdmCl extraction of predentin.

‡ Bovine serum albumin (Sigma A-4378).

§ Abbreviation: nd, not determined.

|| Detected on the amino acid analyser.

¶ Pr, present; —, absent.

** Residues/1000 residues of amino acids.

Fig. 2. SDS/polyacrylamide-gel electrophoresis of fractions from predentin

Electrophoresis was performed in 15% gels at 3 mA/tube with Tris/glycine electrode buffer, pH 8.3, containing 0.1% SDS. The gels were stained with Coomassie Brilliant Blue. Gels: (a) total predentin extract; (b) fraction B₁; (c) fraction B₂; (d) reference standards [from top to bottom: phosphorylase *b* (*M_r* 94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soya-bean trypsin inhibitor (20 100) and α -lactalbumin (14 400)].

On 15%-polyacrylamide/SDS electrophoresis (Fig. 2) it was seen that the material in B₁ corresponded in mobility to the major band seen in the total predentin extract.

The apparent molecular weight and the amino acid analysis of B₁ (Table 1) indicated that this component was in fact bovine serum albumin. To test this possibility, double diffusion was performed with rabbit anti-(bovine serum) IgG. A single sharp precipitation line was obtained at all concentrations. It was thus concluded that albumin was the predominant component of fraction B.

Fraction B₂ contained, in addition to a strong Coomassie Blue-staining band with the same mobility as albumin, only some weakly stainable minor components (Fig. 2). The amino acid analysis of B₂ (Table 1) showed, when the contamination by

Fig. 3. DEAE-Sephacrose chromatography of predentin and dentin extracts

The crude GdmCl predentin extract (15 mg) (a) and dentin EDTA extract (15 mg) (b) were applied to a column (2.6 cm $\times$ 15 cm) of DEAE-Sephacrose equilibrated with 20 mM-Tris/HCl buffer, pH 8.0. The column was eluted at a rate of 50 ml/h with a linear gradient of 0–0.5 M-NaCl over a total volume of 2000 ml. The gradient was interrupted at the elution volume marked by the arrow and flushed with 1 M-NaCl. Chromatography was performed at 4°C and the eluate was continuously monitored for A₂₂₅.

serum albumin was taken into account, the presence of a component high in aspartate and serine.

When fraction B₂ was chromatographed on a Sephadex G-100 column, B₂ was resolved into three components, denoted α , β , and γ (Fig. 4). Fraction γ was found to be identical with bovine serum albumin, depending on its elution position, amino acid composition, mobility on 15%-polyacrylamide/SDS gels and double diffusion against rabbit anti-(bovine serum) IgG. No precipitate was formed when fraction α and β were analysed similarly. Fraction β , however, in spite of its different elution position from bovine serum albumin on Sephadex G-100, had an electrophoretic mobility on 15%-polyacrylamide/SDS gels identical with that of bovine serum albumin and an amino acid composition very reminiscent of this substance (Table 1). Fraction α was found to contain some organic phosphate. The amino acid analysis, showing fairly high amounts of aspartate, serine and glutamate, identify α as being a low-phosphorylated phosphoprotein (PP-L; Linde *et al.*, 1983) containing 4.3% (w/w) phosphate i.e. 26% of the serine residues would be in the form of phosphoserine.

Phosphoprotein, of the type that is generally present in dentin and bone, should have eluted at 0.3–0.4 M-NaCl. Nothing appeared in the chromatogram at this position (Fig. 3a).

Fraction C, eluted with 1 M-NaCl after the

Fig. 4. Chromatography of fraction B_2 on Sephadex G-100

Fraction B_2 (4 mg) from DEAE-Sepharose chromatography of bovine predentin extract (Fig. 3a) was run on a column (1.6 cm $\times$ 95 cm) of Sephadex G-100 (superfine grade) at 4°C. The column was equilibrated with 0.5 M-NaCl in 20 mM-Tris/HCl buffer, pH 8.0, and eluted at a rate of 5 ml/h. The eluate was continuously monitored for A_{225} .

gradient had reached 0.4 M-NaCl, contained large amounts of uronic acid and hexosamine. The ratio between glucosamine and galactosamine was found to be 1:19. The amino acid analysis of fraction C showed high levels of aspartate, serine, glutamate and glycine (Table 1). It was concluded that fraction D contained proteoglycan.

Identical chromatograms were obtained regardless of whether the predentin material was obtained from freshly dissected material or the mandibulae had been frozen previously.

The amino acid analysis of the insoluble collagenous matrix, remaining after the extraction of predentin with GdmCl, is shown in Table 1.

Indirect immunofluorescence was used in an attempt to show the distribution of albumin in predentin (Fig. 5). No specific fluorescence was detected in the predentin matrix. However, fluorescence, indicating the accumulation of albumin, was seen in the predentin tubules.

The dissected dentin was extracted with GdmCl before demineralization with EDTA, thus removing all components not firmly associated with the mineral phase. The chromatogram originating from the EDTA-extracted dentin revealed three major fractions (Fig. 3b). Fraction D, eluting in the void volume, had an amino acid composition clearly demonstrating the presence of collagen (Table 1). No peak was seen at about 0.2 M-NaCl, i.e. corresponding to the elution position of fraction B in the predentin extract. On the other hand, a large

Fig. 5. Localization of albumin in bovine predentin by immunofluorescence

Cryostat sections (3–5 μ m thick) of dissected predentin material were treated for 30 min with rabbit anti-(bovine serum albumin) serum (diluted 1:10) and subsequently washed in phosphate-buffered saline. The section was bathed in FITC-conjugated anti-rabbit IgG (diluted 1:20) for another 30 min and thereafter washed. The sections were mounted and immediately examined in a dark-field fluorescence microscope. Specific fluorescence was located only in predentin tubules and on the surface of the specimens. Magnification $\times$ 712.

peak eluted at about 0.3 M-NaCl. The presence of high amounts of organic phosphate and the very high levels of aspartate and serine in the amino acid analysis (Table 1) showed that this component contained highly phosphorylated dentin phosphoprotein. Fraction F was high in uronic acid and hexosamine. The ratio between glucosamine and galactosamine was identical with that found in the predentin proteoglycan fraction, 1:19.

Discussion

The present study shows that, in addition to collagen, the only major component present in predentin is proteoglycan. A few other components were found to be present in low amounts. No traces were seen of γ -carboxyglutamate-containing proteins as revealed by the DEAE-Sepharose chromatography (Fig. 3a) and the inability to detect such proteins by means of radioimmunoassay in predentin extracts (results not shown). Only very little phosphoprotein was detected. These are major NCP fractions in rat incisor dentin (Linde *et al.*, 1980) and rat bone (Linde *et al.*, 1983). Phosphoprotein is

a major NCP in bovine dentin; other NCPs have, however, not been investigated in dentin from this species.

The absence of other major NCPs from the chromatogram is not due to lack of material. To avoid 'artefactual' degradation and losses of protein, a number of precautions were taken. These included tissue extraction at -130°C , the use of proteinase inhibitors in extraction and demineralization solutions and chromatography in the cold. These techniques have previously been found beneficial in the case of dentin (Linde *et al.*, 1980) and enamel (Termine *et al.*, 1980) NCPs. There is thus every reason to believe that, if other NCPs would have been present in predentin in significant amounts, they would have shown up in the chromatograms. When the dissection of the tissue was carried deeper into the newly formed dentin, the major NCP of dentin, phosphoprotein, appeared in the chromatograms (Fig. 3b).

Small amounts of two types of phosphoprotein were found in the predentin GdmCl extract. It does not seem likely that the phosphoprotein material was derived from contaminating undemineralized dentin, since such components are known not to be extractable from rat dentin by GdmCl without prior demineralization (Linde *et al.*, 1980). The amounts of the highly phosphorylated phosphoprotein (fraction A₂, Fig. 3a) are so low that its presence in the extracts may be explained by the unavoidable contamination of the dissected predentin by cellular components. This is probably not the case with the low-phosphorylated component (fraction α , Fig. 5). Even if this is a minor component, the amounts relative to those of the highly phosphorylated component are high compared with their relative amounts in the mineralized dentin. It thus seems that a low-phosphorylated phosphoprotein (PP-L; Linde *et al.*, 1983) might be a constituent of predentin matrix. The unusual chromatographic behaviour of these components on DEAE-Sephacel is difficult to explain. One possibility is that the phosphorylated proteins become electrostatically associated with soluble collagen and albumin respectively.

Serum components have previously been identified in dentin (Thomas & Leaver, 1977; Kinoshita, 1979; Butler *et al.*, 1981). The major proteinaceous component, in addition to proteoglycan, isolated from predentin in the present study, was albumin. However, immunohistochemistry was unable to demonstrate its presence in the predentin matrix; it was instead found in predentin tubules. Albumin is present in very high concentrations in serum, and it is hence highly probable that albumin is primarily a contaminant from serum, derived from disrupted blood vessels in and beneath the odontoblastoma and included in the predentin scrapings during dissection.

Weinstock & Leblond (1973) demonstrated in an autoradiographic study that [^{32}P]phosphate reached dentin posterior to the mineralization from 90 min after intravenous injection, whereas the collagen precursor [^3H]proline, after passing the odontoblast layer, remained in predentin without entering dentin for at least 4 h (Weinstock & Leblond, 1973). It was concluded that the phosphoprotein passes rapidly between collagen fibres to reach the predentin/dentin junction. Since very small amounts of phosphoprotein were present in the bovine predentin extracts, a more plausible interpretation would be that the phosphoprotein is rapidly transported intracellularly in the odontoblast process and subsequently secreted at the mineralization front. A similar incorporation pattern has been demonstrated for [^3H]fucose (Weinstock *et al.*, 1972). This, together with the present results, implies that other acidic proteins of dentin matrix also follow this intracellular route. The finding of the appearance of an electron-dense material at the predentin/dentin junction (Weinstock & Leblond, 1973) supports this theory. These interesting results may, however, be criticized in that the tissue was demineralized in EDTA. This chelator is known to extract NCPs virtually totally from dentin (Linde *et al.*, 1980).

The pattern that emerges for the proteins of dentin matrix is that of two secretion levels (Linde, 1982). At the first proximal level, collagen molecules and proteoglycans are secreted. The collagen molecules cross-link and form morphologically recognizable fibres. A denser collagen network is thus seen later, i.e. further distally in the predentin, close to the dentin. The role of the proteoglycans is presumably that of a space-filling gel with steric-exclusion properties and as a mineralization inhibitory factor. After having fulfilled this function the amount of proteoglycan decreases considerably and its composition changes (Engfeldt & Hjerpe, 1972; Hjerpe & Engfeldt, 1976). Predentin, and presumably also osteoid, may thus be viewed as similar in composition to normal connective tissue and primarily aimed at the production and maturation of collagen fibres. One exceptional feature is, though, that predentin is devoid of any fibronectin (Linde *et al.*, 1982), a ubiquitous component of collagenous connective tissues. As far as we know at present, predentin has essentially nothing in it that indicates that it is destined to mineralize.

The major part of NCPs is presumably transported intracellularly in the odontoblast process through the predentin zone and secreted at the second level, immediately before the mineralization front. Although the biochemical characteristics of the NCPs have not been studied in detail, it is believed that these initiate, govern and propagate the formation of calcium phosphate mineral (Linde, 1982). Among these NCPs are the phosphoproteins.

The highly phosphorylated phosphoproteins have a strong Ca^{2+} affinity (Cookson *et al.*, 1980; Zanetti *et al.*, 1981) and the Ca^{2+} -binding properties are such that they could influence mineral formation in a variety of modes (Thomas & Leaver, 1977; Linde, 1982).

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