RADIOAUTOGRAPHIC VISUALIZATION OF THE DEPOSITION OF A PHOSPHOPROTEIN AT THE MINERALIZATION FRONT IN THE DENTIN OF THE RAT INCISOR

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A peptide that is rich in organically bound phosphorus and contains abundant serine residues has been identified in the dentin of man (1), fetal bovine (2, 3), and rat (4). This phosphoprotein may play a role in mineralization (5–9). Butler et al. (4) reported that the phosphoprotein of rat incisor dentin constituted 10.8% of the proteinaceous material recovered from decalcified incisor teeth while collagen comprised 84%. Since the phosphorus content of the phosphoprotein was estimated at 3.29% and that of collagen at 0.19% (4), much of the phosphorus taken up in organic form by the incisor would likely be present as phosphoprotein. With this in mind, it was decided to inject [^{33}P]phosphate into rats and examine the demineralized incisor teeth by radioautography in the hope of tracing phosphoprotein formation.

The organic phosphorus of dentin phosphoprotein is believed to be attached to serine residues (6). In the rat incisor dentin, this amino acid comprises 35 residues per cent of the phosphoprotein and only four residues per cent of the cyanogen bromide peptides of collagen (4). Hence serine also appeared to be a suitable amino acid precursor to trace phosphoprotein formation by radioautography.

Finally, the radioautographic pattern of the deposition of labeled phosphorus and serine was compared to that of [^{3}H]proline. Proline may be used as a precursor to trace collagen, since it makes up 22.0 residues per cent of dentin collagen and only 2.4 residues per cent of the phosphoprotein (4). The results indicated that the pattern of phosphoprotein deposition into the dentin matrix is strikingly different from that of collagen.

MATERIALS AND METHODS

Sherman rats weighing 30–40 g received an intravenous injection of either [^{33}P]phosphate (2.5 mCi per animal; carrier-free), L-[^{3}H]serine (2.5 mCi per animal; sp act 2.23 Ci/mmol) or L-[2,3-^{3}H]proline (2.5 mCi per animal; sp act 45.7 Ci/mmol). Two animals per time interval for each precursor were sacrificed under ether anesthesia by perfusion of fixative through the left ventricle 5, 10, 20, 30, and 90 min, and 4 and 30 h after injection. After the injection of [^{3}H]serine, however, one animal per time interval was sacrificed after 30 and 90 min, and 4 h. The fixative consisted of 2.5% glutaraldehyde in 0.05 M Sörensen's phosphate buffer with the addition of 0.1% sucrose and 0.5% dextrose. In the experiments using L-[2,3-^{3}H]proline, the fixative employed was 3% formaldehyde (TAAB Laboratories, Emmer Green, Reading, England) in 0.1 M Sörensen's phosphate buffer with 0.1% sucrose added. The final pH of either fixative was 7.2–7.3. After perfusion for 15 min at room temperature the maxillary incisor teeth were immersed in fresh fixative for 2–3 h and demineralized in EDTA for 2 wk at 4°C (10). Although a 2-wk period is sufficient for exhaustive demineralization, some teeth were demineralized for 3 or 4 wk in the experiments conducted with [^{33}P]phosphate. Specimens were washed overnight in 0.15 M Sörensen's buffer, sliced transversely with razor blades into 1-mm thick sections, postfixed for 1–2 h in 1% OsO_4 in 0.1 M Sörensen's buffer, dehy-

drated in acetone, and embedded in Epon. Semithin (0.5 μm) sections were mounted on glass slides, stained with Regaud's iron hematoxylin, and coated with Kodak NTB2 emulsion for radioautography (11).

With the hope of extracting phosphoprotein, maxillary incisor teeth were removed from the maxillae, scraped clean of adhering tissue, and decalcified for 2 wk at 4°C in 0.5 M acetic acid before fixation. The teeth were then rinsed for 3 h in three changes of distilled water, sliced transversely into 2-mm thick sections, and placed for 5 days at 0–4°C in a solution of 0.5 M Tris-HCl buffer, pH 7.4, containing 1.0 M NaCl (4). This is referred to hereafter as Tris-NaCl buffer. Control tissues, decalcified in acetic acid for 2 wk as above, were placed in distilled water for 5 days. Experimental and control tissues were rinsed overnight in distilled water, fixed in 2.5% glutaraldehyde, and embedded in Epon as outlined above. Thin sections of the predentin-dentin junction were stained with uranyl acetate plus lead citrate (12) and examined in a Siemens Elmiskop I.

RESULTS

[^{33}P]*Phosphate*

Since the teeth had been demineralized, the radioautographic reactions indicated the presence of organically bound phosphorus. The odontoblast was the site of [^{33}P]phosphate uptake, as observed 5–10 min after the injection. Silver grains predominated over the Golgi region, although a few were present over the rest of the cytoplasm and over the nucleus (Fig. 1). By 30 min, silver grains were not only over the cells but also over the odontoblast processes and predentin (Fig. 2). At 90 min, the number of grains over the cells and predentin diminished, but a band of silver grains appeared over the dentin side of the predentin-dentin junction (Fig. 3). The band was accentuated at 4 h (Fig. 7). This pattern of [^{33}P]phosphate uptake and deposition at the edge of dentin was observed in all animals examined.

Two of the animals examined 5–10 min after injection showed radioactivity not only in the odontoblasts, but also at the edge of dentin. Since the material was demineralized, some [^{33}P]phosphate may have been incorporated directly into the dentin matrix as organically bound phosphorus.

[^{3}H]*Serine*

30 min after [^{3}H]serine injection, silver grains were observed over the odontoblasts, principally in association with the Golgi region, but grains were also detected over odontoblast processes and proximal predentin (Fig. 4). By 90 min, most of the radioactivity had left the cells. Silver grains were seen over the proximal predentin and, in addition, a narrow band of grains appeared over the edge of dentin (Fig. 5). At 4 h, abundant silver grains overlay both the middle region of predentin and the dentin side of the predentin-dentin junction (Fig. 6).

[^{3}H]*Proline*

At 5 min after [^{3}H]proline injection, silver grains were observed over the ergastoplasm of the odontoblasts, and, soon thereafter, over the Golgi apparatus (13).[1] At 90 min, label appeared principally over predentin, and at 4 h (Fig. 9), its accumulation in that region was greatest in mid-predentin. Neither at 90 min nor at 4 h were silver grains present over the dentin side of the predentin-dentin junction, in contradistinction to the findings after the injection of [^{33}P]phosphate (Fig. 7) or [^{3}H]serine (Fig. 8).

Electron Microscopy

Ultrastructural examination of demineralized rat incisors in the region of the predentin-dentin junction revealed a sharp line of demarcation between predentin and dentin (Fig. 10). In predentin, typical collagen fibrils were seen in various planes of section; they were free of extraneous material. On the dentin side of the junction, however, electron-opaque particles became associated with the surface of collagen fibrils (Fig. 10). The particles had a diameter varying from 6 to 20 nm and were connected by fine filaments (Fig. 10, *inset*). Collagen fibrils appeared to acquire this material as they passed from predentin into dentin (Fig. 10, black arrowhead).

When teeth were treated with Tris-NaCl buffer before fixation with glutaraldehyde, the line of demarcation between predentin and dentin was no longer evident (Fig. 11). Collagen fibrils appeared unaltered, but over dentin they were free of the electron-opaque particulate material (cf. Figs. 10 and 11). This material was present in control

[1] Weinstock, M., and C. P. Leblond. Synthesis, migration and release of collagen precursors by odontoblasts as visualized by radioautography after [^{3}H]proline administration. In preparation.

tissues which were not treated with Tris-NaCl buffer.

DISCUSSION

In the incisor tooth of the young rat, odontoblasts lie adjacent to a layer of nonmineralizing predentin, beyond which mineralized dentin may be found. With the continuous apposition of new predentin by odontoblasts, older predentin concomitantly transforms into mineralized dentin, resulting in a constant thickness of predentin. Mineralization occurs rather abruptly in this tissue since there is a sharp demarcation line between older predentin and dentin.

In 1959, it was demonstrated by radioautography after the injection of [^{3}H]glycine that odontoblasts released newly synthesized collagen into predentin by 4 h. However, not until the 35-h interval was this labeled collagen observed within dentin (14). The present results after [^{3}*H*]*proline* injection confirmed at 4 h that the label was exclusively in predentin and indeed none had reached dentin proper (Fig. 9). On the basis of the data of Butler et al. (4), it may be calculated that 98 residues per cent of proline present in dentin matrix are within collagen, while only two residues per cent are within the phosphoprotein. Assuming that the proline content of these two substances provides a rough index of the respective amounts which they should take up in the course of dentin formation, almost all of the [^{3}H]proline should be incorporated into dentin collagen. The restriction of the label to predentin demonstrated in this study indicates that newly formed collegen accumulates only in predentin at the 4-h interval.

The pattern of incorporation of the two other labeled precursors was quite different. Notably, as early as 90 min and more clearly at 4 h, much of their radioactivity had appeared on the dentin side of the predentin-dentin junction (Figs. 3, 5–8).

In the case of labeled *serine*, the radioautographic findings at 30 min were similar to those obtained with proline at that time; however, at 4 h the labeled serine was found in both predentin and the dentin side of the predentin-dentin junction (Fig. 6). Since it can be calculated from reference 4 that 48 residues per cent of matrix serine are in collagen and 52 residues per cent in phosphoprotein, the radioactivity may be attributed to incorporation into either collagen or phosphoprotein. At 4 h labeled proline has not yet reached the dentin side of the predentin-dentin junction, so that the newly-synthesized collagen is restricted to predentin. Therefore, the labeled serine found beyond the predentin-dentin junction must be within the phosphoprotein. With regard to the radioactivity in predentin, it may be within either collegen or phosphoprotein.

With labeled *phosphorus*, a radioactive band was observed over the edge of dentin in two animals

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FIGURES 1–3 Light microscope radioautographs prepared after the administration of [^{33}P]phosphate. × 1,000.

FIGURE 1 5 min after ^{33}P injection (12-day exposure), silver grains may be seen over the cells (*Od*), with a predominance of grains over the Golgi region (*G*). The predentin (*Pd*) and dentin (*D*) are unlabeled.

FIGURE 2 30 min after ^{33}P injection (6-day exposure), silver grains are present over the predentin. Label persists in the cells.

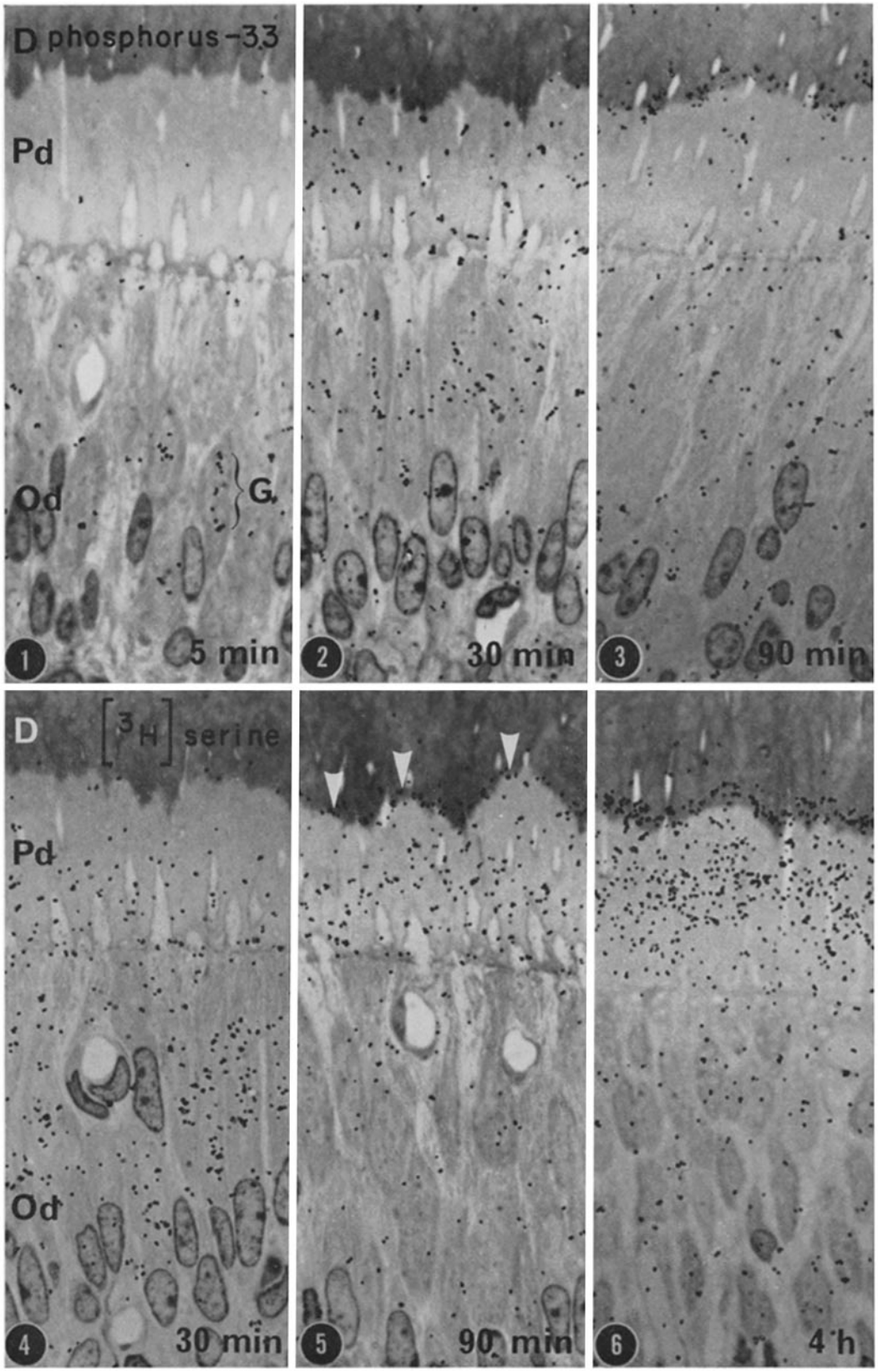
FIGURE 3 By 90 min after injection of ^{33}P (9-day exposure), a band of silver grains may be seen just beyond the predentin-dentin junction.

FIGURES 4–6 Light microscope radioautographs after the administration of [^{3}H]serine. × 1,000.

FIGURE 4 30 min after [^{3}H]serine injection (5-day exposure), silver grains are present over the proximal predentin (*Pd*) and the odontoblast processes. Grains are also localized over the supranuclear region of the odontoblasts (*Od*) corresponding to the Golgi apparatus. Dentin (*D*) is unlabeled.

FIGURE 5 90 min after [^{3}H]serine injection (5-day exposure), silver grains are present along the predentin-dentin junction (white arrowheads) and over predentin, chiefly in the proximal region.

FIGURE 6 4 h after [^{3}H]serine injection (5-day exposure), a band of silver grains is concentrated over the dentin side of the predentin-dentin junction, which is also the region where the labeled phosphorus accumulates(compare with Fig. 7). Grains are also abundant over the middle region of the predentin.


D phosphorus-33
Pd
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G
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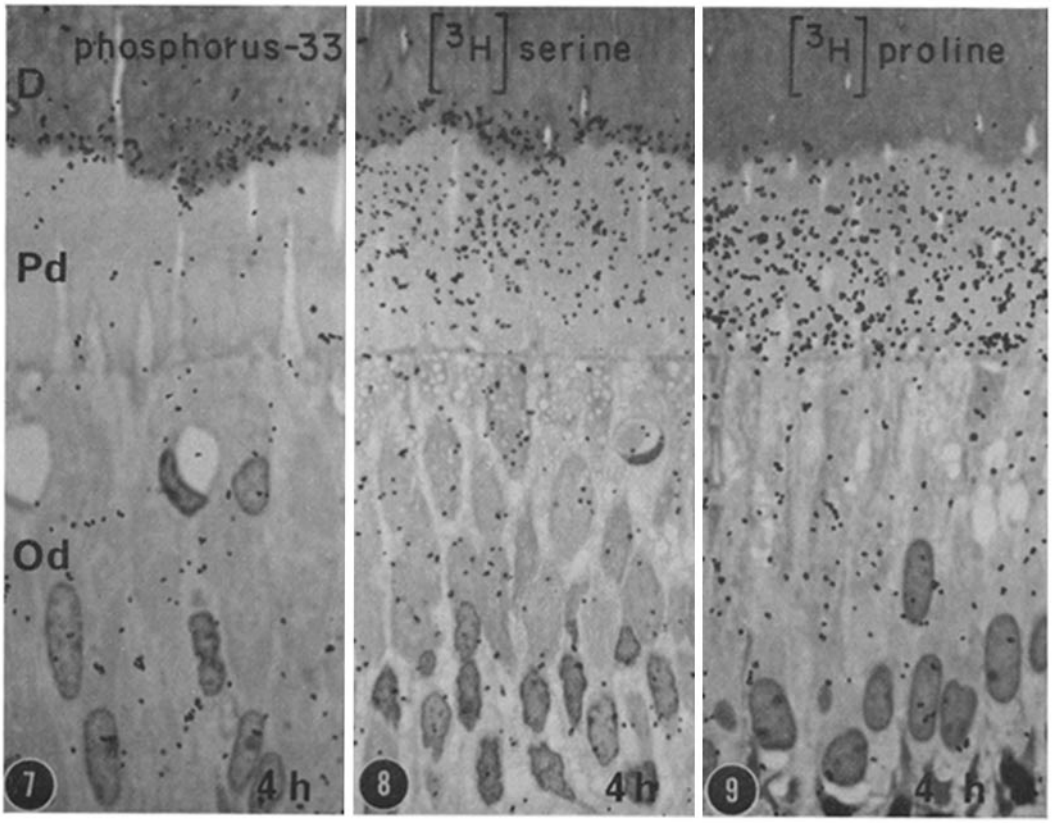
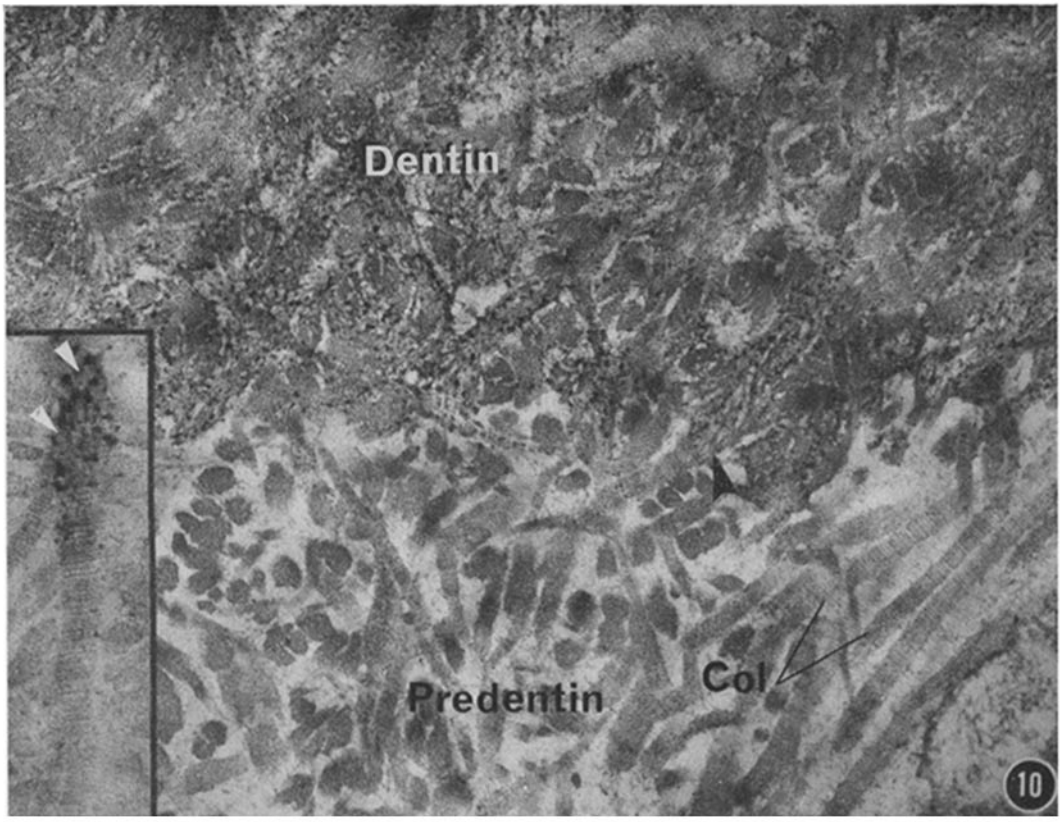


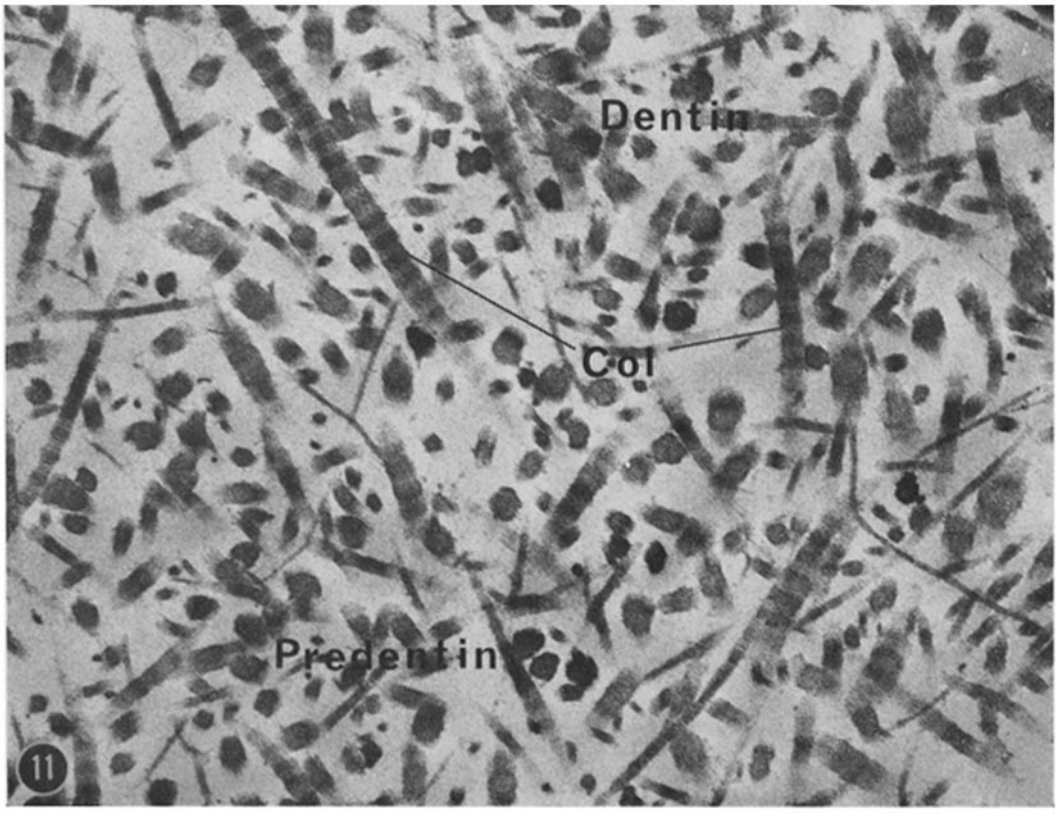
FIGURES 7–9 Light microscope radioautographs comparing the 4-h time interval after the administration of [^{33}P]phosphate (6-day exposure), [^{3}H]serine (5-day exposure) and [^{3}H]proline (3-day exposure). × 1,000. After the injection of [^{33}P]phosphate (Fig. 7) and [^{3}H]serine (Fig. 8), a preponderance of silver grains occurs as a distinct band on the dentin side of the predentin-dentin junction (mineralization front). On the other hand, after the injection of [^{3}H]proline the preponderance of silver grains is over the predentin. A band of radioactivity is not seen at the mineralization front (Fig. 9).

5 and 10 min after injection, that is, at the location of the mineralization front. Although the label may indicate the presence of some radioactive inorganic phosphate which had not been removed by decalcification, such an interpretation is unlikely since the teeth were kept in EDTA for periods of up to 4 wk, which is more than ample time for complete demineralization (10). Furthermore,

FIGURE 10 Electron micrograph of the predentin-dentin junction in the rat incisor. Naked collagen fibrils (*Col*) may be seen in the predentin. At the junction and beyond within the dentin, collagen fibrils possess a coating of electron-opaque particles and filaments on their surface. Fibrils appear to acquire this material as they traverse the junction from predentin to dentin (black arrowhead and *inset*). The coating is composed of electron-opaque particles apparently linked by fine strands (white arrowheads in inset). This material likely corresponds to the phosphoprotein of dentin. × 60,000; *inset*, × 70,000.

FIGURE 11 Electron micrograph of a region of the predentin-dentin junction in the rat incisor after decalcification and treatment with Tris-NaCl buffer. The interface between predentin and dentin is no longer present. Collagen fibrils (*Col*) appear intact and unchanged by the treatment (compare with Fig. 10). Fibrils within dentin no longer possess an electron-opaque material on their surface. × 60,000.


Dentin
Predentin
Col
10


Dentin
Col
Predentin
11

when paraffin sections of EDTA-demineralized tissue were treated with 5% nitric acid before radioautography, there was no apparent reduction in the number of silver grains at the mineralization front. Hence, a more plausible explanation is that this label represented organically bound phosphorus. Perhaps some labeled phosphorus was exchanged with, or covalently linked to, a substance located at the mineralization front. In this regard, it is of interest that an enzyme capable of phosphorylating the serine residues of proteins has been isolated from mineralizing tissues (15).

While direct [^{33}P]phosphate uptake by dentin was observed in only two animals, the presence of organically bound phosphorus in odontoblasts at early intervals (Fig. 1) and the subsequent migration of newly synthesized phosphoprotein to the dentin side of the predentin-dentin junction was noted in all of them. It was again calculated (4) that 69% of the organically bound phosphorus of dentin is within phosphoprotein and the remainder in collagen. Judging from the location of the proline label, no newly formed collagen had reached the edge of dentin by 4 h. Therefore, the radioactive band found at 90 min and 4 h over the edge of dentin may be attributed to the accumulation of newly synthesized phosphoprotein.[2] The presence of both labeled serine and labeled phosphorus on the dentin side of the predentin-dentin junction as early as 90 min after their injection indicated arrival of the phosphoprotein. Hence, over this period of time, the phosphoprotein had not only been synthesized and secreted by the odontoblasts into the predentin, but also had crossed the entire thickness of predentin to reach the edge of dentin. It may be concluded that (*a*) odontoblasts synthesize the collagen and phosphoprotein of dentin; (*b*) both are deposited into predentin; and (*c*) while the collagen accumulates in predentin, the phosphoprotein passes between collagen fibers to reach the predentin-dentin junction where it accumulates by 4 h.

[2] Although phospholipids, including phosphatidylserine, are present in fetal bovine dentin (16), autoradiographic detection of the incorporation of [^{3}H]-serine and [^{33}P]phosphate into these substances is unlikely since phospholipids comprise only 29.2 mg per 100 g of dried demineralized dentin and, therefore, constitute a small amount of the total organic matrix, probably in the form of lipoprotein complexes (17). It is of interest that Irving (18) demonstrated histochemically the presence of sudanophilic material in the region of the mineralization front.

The passage of labeled fucose to the mineralization front was recently observed in rat incisor dentin and it was suggested that a glycoprotein accumulates at that site (19). On the basis of unpublished biochemical data obtained in our department by A. Rizzo, the fucose containing glycoprotein is distinct from the phosphoprotein isolated by Butler et al. (4). Both substances apparently accumulate at the mineralization front at 4 h.

Examination of decalcified specimens in the electron microscope showed an electron-opaque particulate material associated with the surface of the collagen fibrils of dentin including those located at the mineralization front (Fig. 10). When the teeth were treated with the Tris-NaCl buffer used in biochemical experiments to extract the dentin phosphoprotein (4), the particulate material was removed from the surface of the collagen fibrils (Fig. 11). These findings strongly suggest that the electron-opaque particulate material corresponds to the phosphoprotein identified in dentin by biochemical procedures. If this were indeed the case, our results would indicate a close association between the phosphoprotein and the collagen fibrils in dentin. However, from these observations alone, it cannot be stated whether the bond between these two substances is a covalent one as suggested by studies on fetal bovine dentin (3, 6) or a more labile one as in the case of rat incisor dentin (4).

It is known that, in bone (7) and dentin (20), collagen fibrils provide a substrate for the deposition of calcium phosphate minerals. However, minerals are not taken up by the collagen-rich predentin. They are deposited at the mineralization front, that is, at the site where collagen fibrils acquire the particulate material tentatively identified as phosphoprotein. This observation supports the suggestion made by others (5–8) that the phosphate groups of phosphoprotein serve as nucleation centers for mineralization by providing reactive groups for the addition of calcium ions.

SUMMARY

The formation of dentin phosphoprotein by odontoblasts and its deposition into the matrix were studied by radioautography using the precursors [^{33}P]phosphate and [^{3}H]serine, and the results compared with those obtained after [^{3}H]proline injection to trace collagen formation.

Within 30 min, labeled phosphorus, serine, and proline are taken up by odontoblasts and deposited into predentin. The proline label remains in predentin until at least 4 h after injection, whereas

labeled phosphorus and serine are displaced to the dentin side of the predentin-dentin junction, that is, the mineralization front, as early as 90 min after injection. An electron-opaque material associated with the surface of collagen fibrils at the mineralization front has also been identified and is believed to represent the morphological counterpart of the phosphoprotein.

In conclusion, odontoblasts synthesize both collagen and a phosphoprotein. While the collagen accumulates in predentin, the phosphoprotein passes through predentin to reach the mineralization front, where it may play a role in mineral deposition.

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