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Dental Phosphoprotein-Induced Formation of Hydroxylapatite during in Vitro Synthesis of Amorphous Calcium Phosphate†

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ABSTRACT: (Ethylenedinitrilo)tetraacetic acid soluble phosphoproteins were isolated from rat incisor and bovine unerupted teeth. This material was examined for its effect on the stability of amorphous calcium phosphate in vitro. When the precipitation of amorphous calcium phosphate was attempted in the presence of small amounts of these phosphoproteins, an apatite-like mineral was observed to form, which was approximately 60% crystalline, as determined by infrared measurements. This apatite phase could not be induced by addition of phosphoprotein after the precipitation reaction. The organic phosphate bound to these phosphoproteins was shown to be directly responsible for the formation of the apatite phase, since removal of 60% of the covalently bound phosphate with alkaline phosphatase destroyed the protein's ability to induce hydroxylapatite formation. The properties of the dental phosphoproteins appear to be consistent with their possible involvement in the development of the mineral phase of dentine.

The mineral component of bone and teeth has been shown to consist of two phases, a hydroxylapatite-like crystalline phase and an amorphous calcium phosphate phase (Harper and Posner, 1966; Termine and Posner, 1966). Moreover, the crystalline phase increases with age from a value of 30% in femurs of 8-day-old rats to 65% in 26-day-old rats (Termine and Posner, 1966; Posner, 1973). It has been suggested that the earliest mineral to be deposited in bone and dentine matrices is the amorphous fraction (Termine and Posner, 1967). With maturation, the amorphous content of these tissues is reduced to a limiting value of about 10%, although a somewhat disordered carbonate containing analogue of hydroxylapatite may also be present (Posner and Betts, 1975). In order to characterize the amorphous component of bone mineral, extensive investigations have been carried out on synthetic ACP[1] (Posner and Beebe, 1975; Posner and Betts, 1975). The ACP formed under basic conditions is believed to be similar to that which occurs biologically (Betts et al., 1975; Posner and Betts, 1975).

The organic components of bone and dentine matrices may play an important role in the stabilization or modification of the mineral phase. A number of laboratories have reported the occurrence of phosphoproteins in bones and teeth (for a review see Leaver et al., 1975). The nature of the phosphoprotein fraction isolated appears to depend on the type of extraction used, that is, whether acid or neutral, degradative or nondegradative (Dickson et al., 1975). However, one characteristic of this protein fraction that is consistently observed is its high content of aspartic acid (>30% residue) and serine (>30% residue, some of which is *O*-phosphoserine). Recently, experiments by Weinstock and Leblond (1973) demonstrated the rapid appearance of serine and phosphate at the mineralization front of the rat incisor, strongly supporting the early suggestion by Veis and Perry (1967) that dentine phosphoprotein is involved in mineralization. The studies reported here were undertaken to further define the role that dentine phosphoprotein may play in mineralization and, specifically, to determine its effects on the formation of hydroxylapatite.

Experimental Procedure

Unerupted bovine teeth were obtained from the lower jaws of cattle between 1 and 2 years of age. All operations were carried out at 4 °C. After removal from the dental sacs, the teeth were fractured, freed of pulp, and repeatedly washed with cold 0.45 M NaCl. A 24-h soak in 7 M guanidine hydrochloride (pH 7) removed the last remnants of soft tissue. After thorough washing with distilled water, the teeth were extracted with 1.5 volumes of 0.35 M EDTA (pH 8.1) four times over a period of 11 days. The combined EDTA extracts were dialyzed against distilled water until free of calcium and lyophilized.

Incisors were taken from 130–140 g male Wistar rats. After removing the apex, the tooth was freed of pulp and washed

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[1] Abbreviations used are: ACP, amorphous calcium phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl; NBS, National Bureau of Standards.

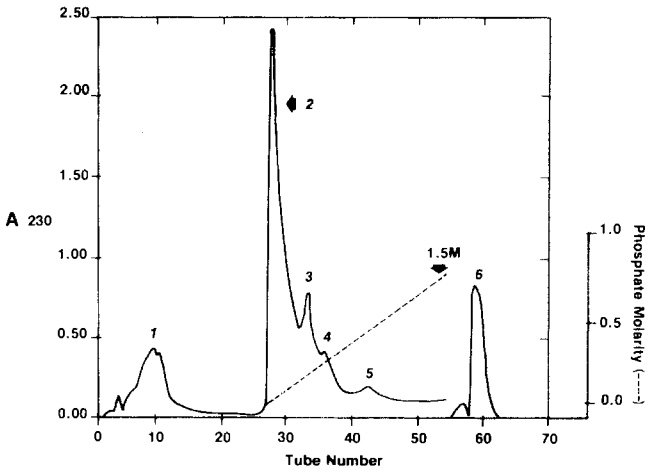

FIGURE 1: Hydroxlapatite chromatogram of the EDTA extract of rat incisor dentine. A 99-mg sample was applied to a 1.0 × 29 cm column in 1 mM phosphate buffer (pH 6.8) and elution was carried out at room temperature. The broken line indicates the actual phosphate gradient used for elution, as determined by refractometry. The arrow indicates the point of application of a final 1.5 M phosphate wash. The column was monitored at 230 nm and 7-ml fractions were collected.

thoroughly with cold 0.15 M NaCl. The extraction with EDTA was identical to that used for the bovine teeth. However, when the enamel visibly separated from the dentine, the dentine was removed and placed into fresh EDTA. Only the EDTA extract after this point (usually two additional changes) was saved. While this separation of dentine and enamel was not thorough, gross contamination by enamel proteins was conveniently avoided.

The EDTA extracts were fractionated on 1.0 × 29 cm hydroxylapatite columns (Hypatite C, Clarkson Co., Williamsport, Pa.) at room temperature. Approximately 100-mg samples were applied in 25 ml of 1 mM sodium phosphate (pH 6.8), which was prepared in 3 M deionized urea. After an additional wash with 100 ml of 1 mM buffer, a linear phosphate gradient of 250 ml from 1 mM (pH 6.8, 3 M urea) was used for elution. A final wash with 1.5 M phosphate (3 M urea) was found to elute a major phosphoprotein fraction. When only this last component was desired, gradient elution was conveniently replaced by a two-step elution: first with 1.0 M phosphate, followed by the 1.5 M buffer. Column fractions were dialyzed exhaustively against distilled water until free of inorganic phosphate and lyophilized for storage.

A 9.0-mg portion of bovine phosphoprotein was dephosphorylated with agarose-bound alkaline phosphatase (25 units, Sigma Chemical Co., St. Louis, Mo.) in 12 ml of 0.15 M Tris buffer (pH 8.0). The reaction was carried out for 4 h at 37 °C.

Purified, reconstituted, acetic acid-soluble rat tail tendon collagen used in one study was prepared according to the procedure of Piez et al. (1963). Crystalline phosvitin was purchased from Sigma Chemical Co., and was purported to contain 8–10% phosphorus.

The Tris-buffered system (pH 8.0) of Boskey and Posner (1973) was used to synthesize ACP at room temperature. The product was quickly washed with cold acetone, dried, and stored desiccated in vacuo. At this pH, the synthesis, if completed in less than 30 min, gave a product that was amorphous, as judged by x-ray and infrared techniques given below. Phosphoprotein and other materials were incorporated into this synthetic scheme by dissolving a known amount of protein in the prebuffered dibasic sodium phosphate prior to the addition of the calcium chloride solution. The amount of the test substance added was expressed as mg/100 ml of phosphate buffer. Agents examined in this way were: (1) bovine dentine and rat incisor phosphoproteins, (2) partially dephosphorylated phosphoprotein, (3) hydroxylapatite that had been synthesized by the method of Tiselius et al. (1956), (4) acetic acid-soluble rat tail tendon collagen, and (5) crystalline phosvitin. It should be emphasized that, except for the addition of these substances, the synthesis was identical to that used by Boskey and Posner (1973) and, in all cases, the mineral was harvested within 30 min. As a control, the synthesis of ACP was checked repeatedly in the absence of any additives.

The synthesis of magnesium-stabilized ACP was carried out according to the procedure of Boskey and Posner (1974) in which magnesium ion is included in the calcium chloride solution to the extent of 20 atom %. Magnesium chloride was prepared by the addition of HCl to MgO.

The apatitic nature of the minerals formed in the presence of phosphoprotein was established with a Philips XRG-3000 x-ray diffractometer equipped with Ni-filtered Cu radiation. Spectra were compared to that obtained with a National Bureau of Standards hydroxylapatite (courtesy of Dr. W. Higuchi, The University of Michigan).

A Beckman IR-10 or Acculab 6 was used to obtain infrared spectra of all mineral samples in the region of 450–650 cm^{-1}. Samples were first exhaustively mixed in an agate mortar and pestle to insure representative sampling. KBr pellets were then prepared at a concentration of 1–2 mg of calcium phosphate/200 mg of KBr. The antisymmetric bending frequency of the phosphate ion near 600 cm^{-1} was used to estimate the percent crystallinity in the samples. The splitting function of Termine and Posner (1966) was calculated for samples exhibiting significant crystallinity. For such quantitation, spectra obtained with 1% (w/w) KBr pellets were used. A series of ACP–hydroxylapatite mixtures of known composition were prepared by weight from the pure, dry components for comparison.

Polyacrylamide gel electrophoresis was carried out in Tris buffers according to the procedure of Davis (1964). Amino acid analyses were performed on a Jeol 6 AH analyzer using a one-column method or on a Beckman 120c, after hydrolysis with 6 N HCl in evacuated tubes at 108 °C for 22 h. Phosphate was determined using the procedure of Chen et al. (1956). The phenol–sulfuric acid assay of Keleti and Lederer (1974) was used to determine carbohydrate content.

Results

The phosphoproteins in the EDTA extracts of the teeth were separated from other materials on columns of hydroxylapatite. It has been reported that phosphoproteins have a high affinity for hydroxylapatite (Bernardi, 1971). A chromatogram of an extract of rat incisors is shown in Figure 1. Extracts of bovine teeth gave similar chromatograms, with the exception that peaks 3 and 4 were not well separated from peak 2 and the material corresponding to peak 5 was missing. Under the conditions employed, recovery of material from these columns was complete. The early fractions of Figure 1 elute in the order of increasing content of acidic amino acids, aspartic and glutamic acids, and are similar to proteins recently obtained under similar conditions from chicken bone (Hauschka et al., 1975). The amino acid compositions of fractions 1, 2, 3, and 5 are included in the table of the supplementary material to this report.

Of particular interest is the component eluted with 1.5 M phosphate (peak 6). Similar materials were observed in the EDTA extracts from both rat incisor and bovine teeth. The

TABLE I: Amino Acid Compositions of Hypatite C Fractions Eluted with 1.5 M Phosphate.[a]

	Rat Incisor[b]	Bovine Teeth
Lysine	11.8	52
Histidine	8.6	6.1
Arginine	5.7	2.4
Hydroxylysine	-	Tr
Hydroxyproline	-	-
Aspartic acid	367	382
Threonine	14.6	8.6
Serine (+ phosphoserine)	421	456
Glutamic acid	59	18.5
Proline	14.4	Tr
Glycine	54	40
Alanine	22	10.3
Half-cystine	-	-
Valine	5.3	10.2
Methionine	3.3	-
Isoleucine	2.6	2.1
Leucine	7	4.5
Tyrosine	2.7	6.1
Phenylalanine	1	1.6

[a] Values given are residues/1000; Tr, trace. [b] Peak 6, Figure 1.

compositions of the 1.5 M eluates of both extracts are shown in Table I. These materials appear to be identical with the EDTA-soluble phosphoprotein of bovine dentine separated on DEAE-cellulose by Veis and co-workers (Veis et al., 1972; Dickson et al., 1975). The components described in Table I contain approximately 5% organic-bound phosphorus and a small amount of carbohydrate (<1%). The phosphorus is probably bound as *O*-phosphoserine. This tentative conclusion is based on the following. First, a rather significant peak was found in the position of *O*-phosphoserine in chromatograms obtained on hydrolysates with the amino acid analyzer. This peak contained organic phosphate. Secondly, the low content of threonine and absence of half-cystine residues would suggest that *O*-phosphothreonine and/or acetic acid, if present, make only minor contributions to this peak.

The phosphoprotein fraction, peak 6, isolated from bovine teeth was further examined for purity by polyacrylamide gel electrophoresis. In 5% gels at pH 8.0 and 8.9 only one band was seen. The use of 8 M urea as a solvent did not alter these results. Thus, this fraction appears to represent a single component. Attempts to determine its molecular weight by use of the analytical ultracentrifuge have, thus far, been unsuccessful due to aggregation. In agreement with the observations of Veis et al. (1972) and Dickson et al. (1975), this protein exhibited an A_{260}/A_{280} ratio of 1.8, suggestive of bound nucleotide material. Dickson et al. (1975), have shown that this 260-nm absorbing moiety is absent from the phosphoprotein that is isolated after an acid demineralization step.

The phosphoproteins obtained from both rat incisor and bovine unerupted teeth were examined for their effects on the synthesis of ACP in vitro. In the absence of these proteins, the calcium phosphate synthesized at pH 8.0 by the method of Boskey and Posner (1973) invariably gave x-ray and infrared spectra that are typical of ACP. An infrared spectrum of ACP is shown in Figure 2 where it is compared with that of a standard hydroxlapatite and a 50% (w/w) mixture of ACP and hydroxylapatite. The infrared spectrum of the mineral synthesized at pH 8.0 in the presence of rat incisor phosphoprotein at a concentration of 2.4 mg of phosphoprotein/100 ml of

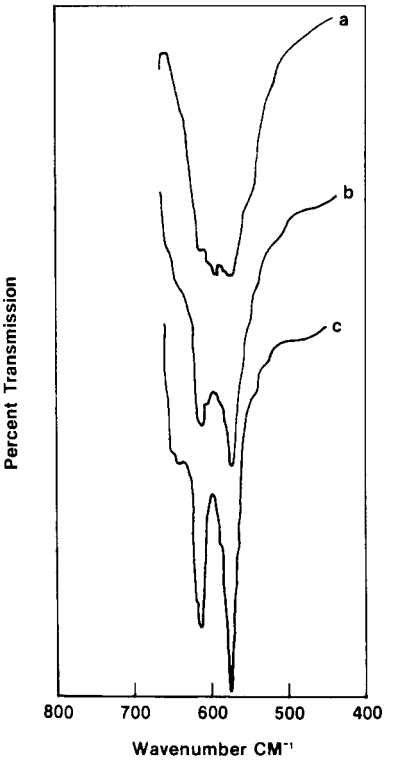

FIGURE 2: Infrared spectra of calcium phosphates: (a) amorphous calcium phosphate, (b) 50 weight % of ACP and hydroxylapatite, and (c) hydroxylapatite.

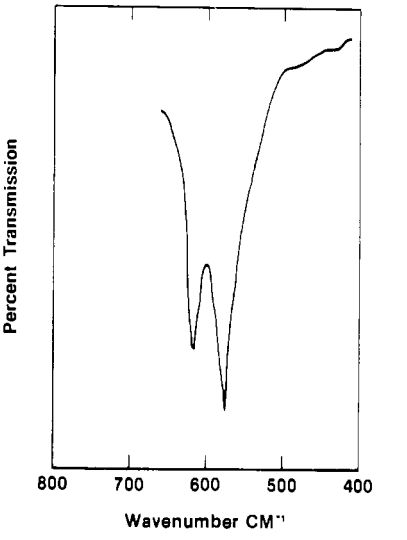

FIGURE 3: Infrared spectrum of calcium phosphate synthesized in the presence of rat incisor dentine phosphoprotein (2.4 mg of phosphoprotein/100 ml of phosphate solution).

phosphate solution is shown in Figure 3. This sample clearly contains a high proportion of a crystalline phase. It should be noted that the amount of phosphoprotein needed to effect hydroxylapatite formation was $1/200$ of the amount of mineral formed by weight. A portion of this sample was kept at room temperature in 1 mM phosphate buffer (pH 8.0). Aliquots removed after 23, 89, and 138 h gave spectra that were essentially identical with that in Figure 3, demonstrating no additionally significant structural change in this time period.

The phosphoprotein from bovine teeth had a similar effect

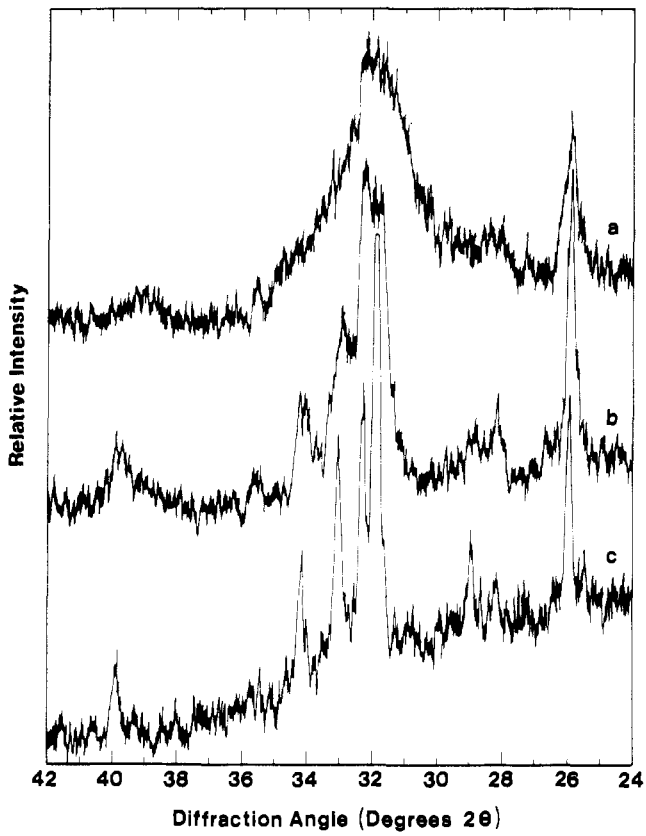

FIGURE 4: X-ray diffraction (Cu Kα) pattern of calcium phosphates: (a) mineral synthesized in the presence of bovine phosphoprotein (2.5 mg of phosphroprotein/100 ml), (b) poorly crystalline hydroxylapatite made by the method of Tiselius et al. (1956), and (c) 100% crystalline NBS hydroxylapatite.

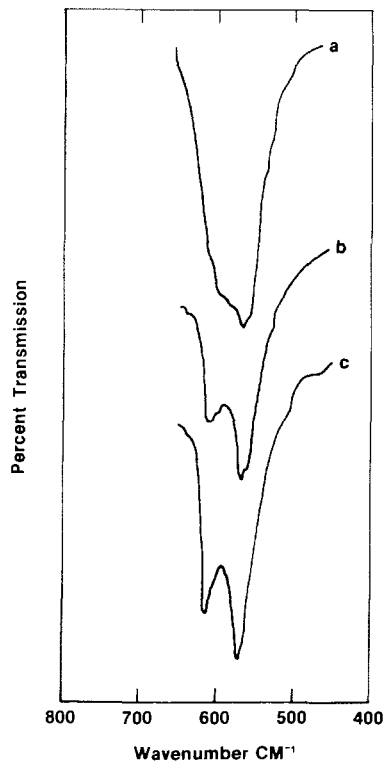

FIGURE 5: Infrared spectra of calcium phosphates synthesized in the presence of various amounts of bovine dentine phosphoprotein: (a) 0.9, (b) 1.2, and (c) 2.5 mg of phosphoprotein/100 ml of phosphate solution.

on the mineral formed during ACP synthesis. The x-ray diffraction pattern of this mineral is shown in Figure 4a. Though poorly crystalline, the material clearly contains an apatite-like phase. Indeed, the diffraction pattern resembles that of a poorly crystallized hydroxylapatite (Figure 4b) or bone mineral, as reported by Harper and Posner (1966).

In order to establish the limits of effectiveness, bovine phosphoprotein was studied over a broad concentration range of 0.9–12.7 mg/100 ml. Results obtained at three different concentrations are illustrated in Figure 5. Below 1 mg/100 ml, the amount of hydroxylapatite induced decreased drastically (Figure 5a). On the other hand, spectra of samples formed at phosphoprotein concentrations higher than 2.5 mg/100 ml were essentially identical to that obtained at 2.5 mg/100 ml (Figure 5c) and, hence, are not reproduced here. Analyses of these minerals for phosphoprotein content gave an estimated range of 0.7–44 μg of protein/mg of mineral. This estimate was based on determination of the aspartic acid content of the minerals and the compositional data of Table I. However, there did *not* appear to be a trend in the aspartic acid content of the minerals. That is, the mineral prepared with the highest concentration of soluble phosphoprotein did not contain the highest amount of aspartic acid. These results seem to correlate with the small amounts of phosphoprotein required for hydroxylapatite induction. It seems likely that the protein remains largely in solution, where further hydroxylapatite formation can be induced. However, some of the phosphoprotein is incorporated into the solid mineral phase, probably by entrapment.

Estimates of the amounts of apatite-like mineral present in the calcium phosphate prepared in the presence of phosphoprotein were made by calculating splitting functions according to the method of Termine and Posner (1966). ACP–hydroxylapatite mixtures prepared by weight gave splitting functions that were in reasonable agreement with those obtained by Termine and Posner (1966). The following linear relation was found between splitting function and weight percent hydroxylapatite using a well crystallized HA:

$$\% \text{ HA (by wt)} = 4.9 \times 10^2 \text{ SF} + 3.5$$

Based on this relation, the calcium phosphates prepared in the presence of phosphoprotein (at concentrations >2.5 mg/100 ml) contained between 50 and 70% hydroxylapatite-like material by weight. This estimate is necessarily approximate, since some variability in the selection of a suitable baseline exists in this region of the infrared. In addition, this estimate is probably low and the actual value may be near 100% hydroxylapatite. Blumenthal and Posner (1972) have shown that the infrared splitting factor of 100% hydroxylapatite increases with increasing crystal size. The ACP samples prepared in this study are of smaller particle size than the hydroxylapatite standard used and, hence, the ir splitting functions would give minimum estimates of percent hydroxylapatite.

A number of experiments were carried out to examine the nature of the effect observed with the dental phosphoproteins. First, the possibility that phosphoprotein effected a rapid conversion of ACP to hydroxylapatite was eliminated by the results of an experiment in which phosphoprotein was added to the ACP slurry 5 min *after* mixing the calcium chloride with the sodium phosphate solution, that is, after precipitation was complete. The mineral was then isolated as usual. Two different bovine phosphoprotein concentrations, 2.7 and 4.0 mg/100 ml, were examined in this way. In both cases, the

precipitated mineral was completely amorphous, indicating that the presence of phosphoprotein is required *during* the precipitation reaction in order for hydroxylapatite to be formed.

The phosphoproteins of dentine origin appear to be unique in inducing hydroxylapatite formation in vitro. Several other materials were examined: (1) hydroxylapatite crystals (1.9 mg/100 ml), (2) reconstituted acetic acid-soluble rat tail tendon collagen (4.0 mg/100 ml), and (3) phosvitin (2.5 and 10 mg/100 ml). None of these substances were able to induce the formation of detectable amounts of hydroxylapatite. Additionally, bovine phosphoprotein was dephosphorylated with alkaline phosphatase to the extent of about 60%, based on inorganic phosphate released and the organic phosphate that remained. This dephosphorylated protein failed to produce an apatite-containing mineral when present at a concentration of 5.9 mg/100 ml. Hence, certain organic phosphate residues appear to be of prime importance in producing apatite. This was also suggested by experiments in which magnesium ions were included in the synthesis to the extent of 20 atom %. Boskey and Posner (1974) had shown that the ACP formed in the presence of at least 20 atom % Mg^{2+} was stable in aqueous solution. When phosphoprotein (2.7 mg/100 ml) was added to this system, no apatite was observed. A reasonable explanation of this observation is that magnesium ions might complex the protein phosphate sites that are essential for seeding hydroxylapatite. Alternatively, the stabilizing influence of magnesium on ACP cannot be overcome by phosphoprotein.

Discussion

Small amounts of phosphoproteins isolated from either rat or bovine dentine induced the formation of hydroxylapatite in an in vitro system that, in the absence of these proteins, yielded exclusively ACP. Based on infrared spectra, a minimum of 60% of the mineral formed in the presence of the phosphoproteins was hydroxylapatite-like. In agreement with Termine and Posner (1970), other molecules, such as collagen and phosvitin or the addition of phosphoprotein after the initial precipitation reaction, did not effect hydroxylapatite formation. If only the total amount of organic phosphate were important, phosvitin, with even more covalent phosphate than the dental phosphoproteins, should have been effective. From experiments in which alkaline phosphatase treated phosphoprotein was used, it is evident that the presence of certain organic phosphate residues in the molecule is required for the induction of hydroxylapatite formation. The solution of alkaline phosphatase-treated protein still contained the organic phosphate concentration that would have been present if untreated phosphoprotein were used at a concentration of 2.4 mg/100 ml, which is known to be effective.

Weinstock and Leblond (1973) have shown the rapid appearance of labeled serine and phosphate at the mineralization front of the rat incisor following intravenous injection. In all probability, the labeled material represents phosphoprotein that has been synthesized and secreted by the odontoblast. The mineral first deposited in the bone and dentine matrix in vivo is likely to be amorphous. In bone, this mineral may derive from matrix vesicles (Anderson, 1969; Bonucci, 1970). In view of the properties of the phosphoproteins noted above, these proteins may be involved in the nucleation in vivo of a hydroxylapatite phase. Experiments are in progress to demonstrate the location of these phosphoproteins in developing teeth and to further study the mechanism of action by which these molecules are able to induce hydroxylapatite formation.

Acknowledgments

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Supplementary Material Available

Amino acid composition data will appear following these pages in the microfilm edition of this volume of the journal (1 page). Ordering information is given on any current masthead page.

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