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Biosynthesis Of Dentin Gamma-Carboxyglutamic Acid-Containing Proteins By Rat Maxillary Third Molars In Organ Culture (Mineralization, Odontogenesis, Connective Tissues, Odontoblasts).

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BIOSYNTHESIS OF DENTIN γ-CARBOXYGLUTAMIC ACID-CONTAINING PROTEINS BY RAT MAXILLARY THIRD MOLARS IN ORGAN CULTURE

by

RICHARD DAVID FINKELMAN

A DISSERTATION

Submitted in partial fulfillment of the requirements for
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ABSTRACT OF DISSERTATION
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Title Biosynthesis of Dentin γ-Carboxyglutamic Acid-containing Proteins by Rat Maxillary Third Molars in Organ Culture

An abundant Ca^{2+}-binding protein containing γ-carboxyglutamate (Gla) has been discovered in bone. The function of this protein remains obscure, but it is thought to be involved in the regulation of mineralization and mineral metabolism. An analogous and apparently heterogeneous group of proteins also containing Gla exists in dentin. Data regarding these proteins are scarce, however. Therefore, the major objective of this research was to gain information concerning the physiological significance of these proteins. A prerequisite goal for this study was the characterization of an in vitro model of dentinogenesis and mineralization.

Maxillary third molars from 11-day-old rats were cultured with or without serum. Molars were incubated without radiolabel or with either $^{45}CaCl_2$ or [^{3}H]-leucine or both for 24 hours at different times for various culture periods. Tooth germs were extracted in acid or guanidine hydrochloride. Molar development was evaluated biochemically by analyzing uptake of radiolabel and histologically by the Von Kossa staining technique. Dentin Gla-containing proteins (DGPs) in extracts were detected by immunologic and chromatographic techniques. DGPs in molar sections were detected by immunolocalization using indirect immunofluorescence with antibodies raised against a highly purified DGP preparation.

Molars from both groups showed a general trend of increasing protein synthesis and continued cuspal development for ten days *in vitro*. Molars cultured with serum displayed histologic evidence for mineralized dentin and enamel and a significant increase in ^{45}Ca-uptake after the sixth day. Molars from 11-day-old rats and molars cultured without serum showed no evidence for the presence of mineralized tissues. [^{3}H]-Leucine-labeled DGPs were isolated and identified from both mineralized and non-mineralized molars. In the non-mineralized molar, DGP antigenicity was seen immunohistochemically in odontoblasts but not in predentin, pre-odontoblasts or at any other site. Antigens in the mineralized molar were localized to odontoblasts and dentin.

These data indicate that rat molars cultured with serum mineralize *de novo* and suggest that rat molar odontoblasts synthesize DGPs concurrently with the elaboration of predentin matrix but independently of mineral deposition. This work extends current knowledge regarding the biosynthesis of Gla-containing proteins and presents an important developmental approach for the study of dentin matrix proteins.

Abstract Approved by: **Committee Chairman** William J. Butler

Program Director R. D. Wells

Date 6/6/86 **Dean of Graduate School** Kenneth L. Roozen

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A dissertation is more than an academic exercise. For me it stands as a marker of my development as a graduate student, as a scientist and health care professional and as a person. Such development cannot be realized alone, however; it was only because of the efforts of many individuals that I was able to find the strength and guidance to reach this goal.

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times and times of great stress. Her strength and inspiration will forever serve as guiding lights for a lifetime of endeavors. She and my daughter, Andrea, are constant reminders that the love of a caring family is life's greatest joy.

Finally, I acknowledge my parents, who gave me my start in life and guided my maturation and growth. Their tireless support through all these years will never be forgotten.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

Figure

Page

APPENDIX 4

INTRODUCTION

The process of biological mineralization is an extremely important one. Mineralization, at least in the physiological sense, can be considered as the deposition of inorganic mineral crystals, chiefly hydroxyapatite (HA), within or around an organic matrix. Mineralization has been studied for many years, but the exact nature of the events that lead to such a site- and time-specific deposition of mineral remains obscure.

Since the organic matrix of mineralized tissues consists of approximately 85-90% type I collagen (1), most early research centered on the possible role of collagen in directing mineral deposition and growth. More recently, however, much of this emphasis has shifted to the other, minor components of the organic matrix, the non-collagenous proteins (NCPs) of mineralized tissues. Several investigators (2,3) have postulated that these NCPs may play a role in mineralization. Supportive experimental data exist. Autoradiographic studies have suggested that both a non-collagenous, phosphate-containing protein (4) and fucose-containing glycoproteins (5) are secreted at the mineralization front and may participate in mineral deposition. A serine- and aspartate-rich phosphoprotein, first isolated in 1964 (6,7), is one of the most studied of the NCPs of dentin, the major mineralized tissue in teeth. Phosphoproteins are the most abundant NCPs of dentin (8) and bind calcium with high affinity (9). Small amounts of phosphoproteins can induce HA formation <u>in vitro</u> (10). Finally, other proteins suggested as having a

role in mineralization on the basis of Ca^{2+}- and HA-binding properties include osteonectin (11-13) and the vitamin K-dependent, γ-carboxyglutamic acid (Gla)-containing proteins of mineralized tissues (14).

Dentin is an excellent model for studies regarding mineralization for two major reasons. The first is the relative simplicity of dentinogenesis: there is a unique structural order and organization manifested in the orientation of polarized odontoblasts (the dentin-forming cells), a non-mineralized predentin and, finally, a mineralized dentin. Secondly, dentin formation represents a purely mineralizing system, devoid of the active processes of resorption and remodeling that are present in bone. The present study describes the development of an in vitro model of mineralization utilizing the process of dentinogenesis within the developing rat molar. This model was then used for studies regarding the possible biological significance of the Gla-containing proteins of dentin.

MINERAL-ASSOCIATED γ-CARBOXYGLUTAMIC ACID-CONTAINING PROTEINS

Following the discovery of Gla in many of the blood coagulation proteins (15), a Ca^{2+}-binding protein containing Gla was discovered in the organic matrix of bone (16,17). This protein, termed bone Gla protein (BGP) (18) or osteocalcin (19), is a major component of bone matrix, representing 1-2% of the total protein (17,19-22). BGP has been sequenced from many species, including human (20), cow (23), chicken (21), monkey (24), swordfish (22), sheep (25) and rat (26) and contains 47-51 amino acid residues (molecular weight = 5,200-5,900) (for recent review, see Hauschka [27]). Comparison of these sequence data has

suggested that the primary structure has been highly conserved over 400,000 years of evolution. BGP has been the subject of extensive investigations, but a definite function for the protein has yet to be established.

Formation of Gla

Gla is an unusual amino acid that is formed by a vitamin K-dependent, post-translational carboxylation of specific glutamyl residues in precursor proteins to γ-carboxyglutamyl residues in resultant proteins (28). Vitamin K-dependent carboxylase activity was first demonstrated in a crude rat liver microsomal preparation (28) and has since been obtained from various tissues from a number of species (for review, see Suttie [29]). However, only that from bovine liver has been purified to any great extent (30,31).

The vitamin K-dependent carboxylase has usually been studied at pH = 7.2-7.4, since its activity is markedly reduced at pH above 8 or below 7 (29). The carboxylase has an absolute requirement for molecular oxygen, vitamin K and CO_2 (32). NAD(P)H and/or a reduced pyridine nucleotide-generating system is also required (33), although the chemically reduced form of the vitamin, the hydroquinone (KH_2), can substitute for these factors (34-36). Neither ATP (35,37) nor biotin (38) are needed for the reaction, and it appears that CO_2 and not HCO_3^- is the reactive species (39).

The exact molecular mechanism of vitamin K-dependent γ-carboxylation has not been completely elucidated, but it appears likely that γ-carboxylation is closely tied to microsomal vitamin K epoxidase activity (40) which converts KH_2 to vitamin K-2,3-epoxide. Both of these activities are located together (41,42), utilize KH_2 as substrate (43) and

have similar activities with vitamin K homologues (44). Except for low CO_2 concentrations (which limit carboxylation but do not inhibit epoxidation [43]), conditions which stimulate or inhibit one reaction have the same effect on the other (45,46). In addition, there is a one-to-one stoichiometry between the amount of Gla and epoxide formed by the reaction (47,48). It seems that the reactions can be uncoupled (43,47), that epoxide formation can continue without concomitant carboxylation, but there are no data supporting the converse relationship of carboxylation without epoxidation (29). A microsomal epoxide reductase activity has been found which can regenerate the vitamin K quinone species (49-51) in a stereospecific manner (52). The above findings have led to the postulation of a cycle of vitamin K species and intermediates in which vitamin K is continually utilized to form an epoxide concomitant to γ-glutamyl carboxylation and then regenerated for additional use (29).

The exact relationship between epoxide formation and substrate γ-hydrogen abstraction is not clear, but one hypothesis is that a vitamin K hydroperoxide, formed as an intermediate in the pathway from KH_2 to the epoxide, plays an important role (53). Direct evidence for such an oxygenated vitamin intermediate is lacking, however, and available data are conflicting (29). Most workers suggest that an oxygenated species is involved in the abstraction of a proton to leave a carbanion on the glutamyl residue (54), although a radical-mediated reaction sequence has also been proposed (55). Available data support the former hypothesis (29), and hydrogen abstraction appears to be stereospecific (56). Free CO_2 can then attack the carbanion to form Gla, consistent with the notion that epoxide formation and carboxylation can be uncoupled.

Chemistry of BGP

BGP is distinguished by the presence of three Gla residues at positions 17, 21 and 24 (20-25). These positions are invariant, although in human BGP Glu-17 is only 9% γ-carboxylated to Gla (this feature may, however, represent an age-related *in vivo* decarboxylation) (20). The disulfide linkage joining Cys_{23} and Cys_{29} is also invariant. Hydroxyproline is present in position nine in many species. The primary structure of bovine BGP is presented in Figure 1.

BGP binds to Ca^{2+} with a dissociation constant (Kd) ≅ 1-2 mM for 2 or 3 binding sites (19,57). Indeed, BGP will inhibit precipitation of calcium phosphate as HA from supersaturated solutions of Ca^{2+} and HPO_4^{2-} (17). This inhibition is dependent on the presence of Gla (57), presumably as a result of the interaction of Gla residues with ion clusters which nucleate these phase transformations (58). Similarly, BGP inhibits the brushite to HA transition *in vitro* (19). The three Gla residues are necessary for Ca^{2+}-binding (57), and Gla-Ca^{2+} interactions mediate BGP's high affinity (Kd ≅ 0.6 μM) for HA, yielding approximately 1 mg protein per 17 mg HA (57). Thermally decarboxylated BGP (3 Gla transformed to 3 Glu residues) eliminates Ca^{2+}-binding in solution, but both decarboxylated and reduced and S-carboxyamidomethylated BGP will still bind a 2-fold excess of HA crystals (57). Although these results indicate that neither the Gla residues nor the disulfide bridge are necessary for crystal binding, it still seems likely that Gla residues are necessary for binding to specific sites; HA-bound BGP is completely protected against thermal decarboxylation (57). The isoelectric point (pI) of native BGP is 4.0; for decarboxylated BGP, the pI = 4.5. As could be anticipated, decarboxylation reduced the electrophoretic

FIGURE 1

Primary structure of bovine BGP[1]

Tyr-Leu-Asp-His-Trp-Leu-Gly-Ala-Hyp-Ala-Pro-Tyr-Pro-Asp-Pro-Leu-Gla[2]-Pro-Lys-Arg-Gla-Val-Cys-Gla-Leu-Asn-Pro-Asp-Cys-Asp-Glu-Leu-Ala-Asp-His-Ile-Gly-Phe-Gln-Glu-Ala-Tyr-Arg-Arg-Phe-Tyr-Gly-Pro-Val

[1]from Price, et al. (23)

[2]Gla = γ-carboxyglutamic acid

mobility of BGP (R_f = 0.52, decarboxylated protein; bromophenol blue, R_f = 1.0; native protein, R_f = 0.57; 20% polyacrylamide gel, pH = 8.9) (57).

Calcium also plays an important role in determining the 3-dimensional structure of BGP. Apo-BGP (devoid of Ca^{2+}) exists primarily in random coil conformation (8% α-helix [4-5 residues], 14% β-sheet and 78% random coil) (59,60). The net charge per protein is -9 at pH = 7.4; decarboxylation reduces net charge to a point between -6 and -7 (57,58), allowing α-helical structure to approach 18%. Increased electrostatic shielding (1.5 M NaCl) permits 15% α-helix, while reduction of the disulfide bridge eliminates all α-helical structure (58,59).

Ca^{2+}-binding induces a conformational change in BGP to 38% α-helix, 8% β-sheet and 54% random coil (59,60). In this conformation, approximately 19 residues exist in α-helix. The three Gla residues are required for this transition; decarboxylated BGP acquires only 8% additional α-helix in the presence of Ca^{2+} (25% of that induced in the native protein) (59). It has been suggested that carboxylation may lead to increased disorder; after Ca^{2+}-binding, BGP may acquire a more ordered structure (apparent α-helix), more equivalent to the Glu-containing protein (61). 1.5 M NaCl does not diminish Ca^{2+}-induced acquisition of α-helix, and the disulfide bond is required for the conformational change (59). However, Ca^{2+}-binding causes no conformational change around the S-S region (60). The environment of at least one phenylalanine (and one tyrosine) changes when Ca^{2+} is bound (60).

For Ca^{2+}-binding, the midpoint of the BGP conformational transition when titrated is at 0.75 mM, almost identical to the Kd for Ca^{2+}-BGP binding. Both processes are non-cooperative (Hill coefficient ≅ 1)

(59). Other metal cations can induce an identical conformational transition, with titration midpoint concentrations increasing in the order $Pb^{3+} < Co^{2+} < Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$ (59).

Spectroscopic studies (58-60) have predicted the following structural features:

1. two α-helical domains:
   a) "Gla helix" (residues 16-25 [chicken], 18-25 [human, cow], containing three Gla residues).
   b) "Asp-Glu helix" (residues 30-41 [human, cow, chicken], with four anionic side chains).
2. probable β-turns at residues 26-29, 12-15 (human, cow, chicken), 5-8 (chicken).
3. Cys-Cys disulfide bridge, residues 23-29 (human, cow, chicken, swordfish), forcing two α-helical segments into opposition (in swordfish, the most primitive BGP studied with one long α-helix [residues 13-33], the S-S bond perhaps bends the helical domain).
4. β-sheet structures at the NH_2^- and COO^- termini may be backfolded at β-turns.

Several interesting features result from charge and side-chain distribution in BGP. The Arg_{20} residue (as well as Arg_{19} [human] and Lys_{19} [cow]) offsets electrostatic repulsion in the Gla helix (58) through interactions of the i ± 1, 2, 3, 4, 7 type (62). As a result the chain in this region probably still has some α-helical character even in the absence of Ca^{2+}, a feature not likely for the Asp_{30}-Glu_{41} region (58). Within the α-helical domains there is regular spacing of

charged and hydrophobic amino acid residues at intervals of 3 or 4 residues. In light of the 3.6 residues/turn structure of the Pauling-Corey (63) α-helix, this spacing creates a distinct anionic surface on one side of the protein and a hydrophobic surface on the other. Such a feature is a fairly common one in proteins (64) and is exhibited by the Gla-rich regions of prothrombin (65).

Binding sites for Ca^{2+} are probably formed by the COO^- groups of Gla residues and by opposing COO^- groups of aspartate and glutamate in the two α-helical domains (58), perhaps by bidentate chelation to form a six-membered ring (66). The periodicity of the α-helix predicts that the three Gla residues in BGP are located such that the Ca^{2+}-binding sites are 5.4 Å apart (59). This spacing suggests that of the Ca^{2+}-Ca^{2+} distances present in the HA crystal lattice (67), only two are compatible for BGP-Ca^{2+}, Gla-mediated binding: (1) Ca_I-Ca_I (5.45 Å, xy plane); and (2) Ca_{II}-Ca_{II} (5.84 Å, xz and yz planes). It is of interest to note that there is approximately one molecule of BGP for each HA microcrystal in bone (17).

Biosynthesis of BGP

The mechanism of BGP biosynthesis remains somewhat controversial. BGP is synthesized in organ cultures of isolated bone (68,69), by osteoblast-like (70) rat osteosarcoma cell cultures (71,72) and by human bone cells (73). Immunoreactive BGP was localized to osteoblasts and osteocytes but to no other cell type (including osteoclasts) in developing calvaria (74), and bone cell microsomes contain a vitamin K-dependent carboxylating system which post-translationally converts Glu residues in peptides to Gla (75). Only cells that actively formed bone matrix during postnatal development in either the rat or chick model

synthesized BGP (76); apparently, BGP is a specific product of cells differentiated with respect to bone formation (77).

It appears that BGP is synthesized initially as an Mr = 9,000 intracellular precursor which is processed to native Mr = 5,800 BGP prior to secretion (71,72), but several groups have claimed that higher Mr precursors may exist. A Mr = 70,000 [^{14}C]-labeled, vitamin K-dependent protein is formed <u>in vitro</u> by bone microsomes and in organ culture when incubated with NaH[^{14}C]O_3 (68). Poorly extractable Gla-containing proteins occur in embryonic bone (14,18,78) which can subsequently be released by proteolysis (14,78). Higher molecular weight Gla-containing species (Mr = 85,000, 35,000, 15,000 and 10,000) have been identified in embryonic chick bone in addition to Mr = 5,670 BGP (79). On the basis of low cross-reactivity, these higher molecular weight species have been reported to be immunologically related to BGP (79). The question regarding high Mr precursors of BGP appears to have been resolved by the construction of a cDNA library and the molecular cloning of BGP from rat osteosarcoma cells (26). The cDNA data, yielding a calculated molecular weight of 9120-9240 for pre-pro-BGP, are consistent with the predicted intracellular precursor described above.

Also presently under debate is the initial biosynthesis of BGP relative to the onset of mineralization in bone. Developmental studies have clearly shown that the amino acid Gla is first seen concomitantly with first mineralization in both chick embryonic long bone (77) and in ectopic bone induced in rat by implantation of demineralized bone powder (78). BGP was unequivocally identified by high-performance liquid chromatography (HPLC) in bone from both 17-day-old chick embryo and from six-month-old fetal calf (14). Some investigators, using a

radioimmunoassay (RIA) for BGP, have disputed the presence of BGP in fetal rat bone (18) and in induced ectopic bone (76), although these findings have been contradicted by others who, in preliminary work, did claim to find BGP in fetal rat bone (80).

BGP is present in serum (81) and results from new biosynthesis rather than from release from bone matrix during resorption (82). It seems likely that BGP is synthesized by osteoblasts (74) and is secreted (72) after intracellular, post-translational modification in a vitamin K-dependent manner (71,72). Addition of the vitamin K antagonist warfarin to culture of osteosarcoma cells caused a specific reduction in the rate of secretion of BGP (83). A presumed intracellular, immunoreactive Mr = 9,000 BGP-precursor (pI = 6.0) appears to accumulate within treated (but not in control) cells (83). The protein is then cleared from serum rapidly, primarily by the kidney, with a half-time of four minutes (82).

Function of BGP

Although an exact function for BGP has yet to be established, several interesting hypotheses have been proposed based on recent experimental results. When BGP was first discovered it was thought that the protein might be involved in mineral formation. However, animals maintained from birth to two months of age on a warfarin-treatment protocol grew normally and had normal bone structure and mineralization even though bone BGP levels were only 2% of normal (84). The only known bone defect to result from vitamin K deficiency was seen in rats maintained on chronic warfarin treatment for eight months; such rats showed excessive mineralization with closure of the proximal tibial growth plate and cessation of longitudinal growth (85). This result and others

(86) have prompted the respective authors to propose that BGP acts to inhibit excessive mineralization (85) and to oppose net Ca^{2+} loss from bone metaphysis (86).

On the other hand, BGP has been suggested as a useful clinical marker for bone metabolism (87,88), bone calcification (86,89) and bone formation (90-92). These results have led to the suggestion that BGP may still have a specific role in bone formation (92).

Another intriguing function proposed (93,94) for BGP is the mediation of the action of the steroid hormone, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. $1,25(OH)_2D_3$ caused serum concentrations of BGP to increase in rat (95) and stimulated the biosynthesis of BGP by rat osteosarcoma (96) and human bone (73) cells. This stimulation of BGP biosynthesis was prevented by actinomycin D or α-amanitin, supporting a model of transcriptional control for $1,25(OH)_2D_3$ and suggesting that the hormone stimulates a transient burst of transcription of the BGP gene (97). The possible relationship of vitamin D and BGP is discussed in further detail elsewhere in this manuscript (98).

Finally, BGP may play a role in bone metabolism by regulating bone forming and/or bone resorbing cells. BGP was found to be chemotactic for breast carcinoma cells, monocytes and osteoblast-like rat osteosarcoma cells (99,100). This effect, localized to the COOH-terminal region, was not disturbed by decarboxylation (99). It has also been reported that BGP-deficient bone implants showed impaired recruitment of osteoclast-progenitor cells (101).

Other Gla-containing Proteins

Gla-containing proteins have been discovered in numerous other sites including atherosclerotic plaques (102-105), kidney (106,107),

calcified heart valves (103,108,109), liver mitochondria (110), renal calculi (111) and calcified skin of dermatomyositis and scleroderma (105). A second Gla-containing protein of bone has been isolated from the acid-insoluble matrix and has been termed matrix Gla protein (MGP) (112). Gla has even been identified in invertebrates, two species of hermatypic corals (113). Such proteins may have marked local functions. For example, vitamin K deficiency in rats caused hypercalciuria that may have had origins in kidney vitamin K-dependent proteins (114). Lastly, Gla-containing proteins have been isolated from rat dentin and are highly homologous to those from bone (115,116). These proteins are described in detail below and elsewhere in this manuscript (117).

TOOTH FORMATION

The developing tooth represents a rather remarkable organ for study in the field of developmental biology. The tooth germ arises from the intimate associations and interactions of several embryonic tissue lines involving complex motions of epithelial and mesenchymal cells; its development depends on the precise control of cell-cell interactions among these tissues (or perhaps within tissues) and of cell-extracellular matrix interactions (with the matrix produced either by the same or a different tissue). The elucidation of the specific mechanisms for genetic and epigenetic controls regulating the processes of histo- and morphodifferentiation and sequential gene expression within the developing tooth organ would have broad implications for both developmental and molecular biology.

Formation of the Dental Papilla

The details of the embryogenesis of the dental papilla have been established in amphibian and avian embryos (118-125). The cells of the

dental papilla derive from the migration of cephalic neural crest, as demonstrated by grafts of neural crest between different species. As such the dental mesenchyme (papilla) has been described as ectomesenchyme. A similar participation of the neural crest in mammalian species is generally accepted; direct data from mammals are lacking, however, although indirect histochemical evidence is supportive (126).

It is not known whether the neural crest cells are determined as presumptive dental cells before or during migration (127). There is evidence that the pathway of neural crest migration determines subsequent cellular differentiation (128) and that this control is mediated by interactions with other cells and extracellular matrix along the route of migration (129,130), as well as by the final site of localization (131,132).

Morphogenesis of the Tooth Organ

Tooth development is classically subdivided into four successive stages based on morphology: (1) dental lamina; (2) bud stage; (3) cap stage; and (4) bell stage (133). These stages, although somewhat arbitrary and borne from early histological observations, represent the expression of histodifferentiation, morphodifferentiation and the terminal differentiation of dental cells, chiefly the odontoblasts and ameloblasts.

Dental Lamina and Bud Stage

The first structure that can be identified as the initiation of tooth development in the oral cavity is the dental lamina (134), representing a thickening of oral epithelium resulting from the influence of the underlying "determined" ectomesenchyme. The controls for determining the site of dental lamina formation have been discussed (135-137)

but remain controversial; it has been suggested (138-140) that developing capillaries or nerves establish the position of the tooth bud, perhaps by guiding the migration of neural crest cells.

It is thought that the only determined "dental" cells in the maxillary and mandibular arches are the cells of the dental papilla, the presumptive pulp (127). Dental mesenchyme was able to induce enamel organ formation in foot pad epithelia with subsequent differentiation of epithelial cells into functional ameloblasts (141-143), but teeth were not formed in the converse association of oral epithelium and either limb or gut mesenchyme. In addition, reciprocal transplant combinations of epithelial and mesenchymal components of incisor and molar tooth germs grew in the usual incisor or molar form according to the origin of the mesenchyme, not the epithelium (141).

The dental lamina continues to proliferate into the underlying mesenchyme to form the primordial tooth bud. Such round or ovoid swellings exist in each dental arch corresponding to the future positions of the teeth.

Cap Stage

As the tooth bud continues to grow, specific tissues within the enamel organ become apparent. Histogenesis of the enamel organ is initiated at this stage (144). The tooth bud assumes a cap appearance with its concave surface overlying the dental papilla and its convex surface facing the oral cavity. The cells lining the convex surface are cuboidal and are termed the outer enamel (or sometimes dental) epithelium; those lining the concave surface are tall columnar and are called the inner enamel (or dental) epithelium (IEE) (144). The cells between the inner and outer enamel epithelia separate and become arranged into

a reticular network, the stellate reticulum, with much intercellular fluid. Often seen projecting from the center of the IEE and toward the dental papilla are densely packed cells termed the enamel knot. There is often a corresponding extension of the enamel knot into the stellate reticulum called the enamel cord. Both structures disappear prior to enamel formation.

Bell Stage

As development and proliferation continue, the enamel organ assumes a classical bell shape. In this stage final histo- and morpho-differentiation take place (143). The IEE is composed of a single layer of tall columnar cells, the pre-ameloblasts, which will differentiate into functional ameloblasts and form the enamel. The enamel organ partially surrounds the dental papilla, the peripheral cells of which, adjacent to the IEE, are termed pre-odontoblasts. These cells will terminally differentiate to odontoblasts and form the dentin.

Differentiation of Odontoblasts

In the early bell stage, the ectomesenchymal cells of the dental papilla are separated from the epithelial cells of the IEE by a basement membrane (143). The basement membrane is at least partially epithelially derived (145-148), although its organization may be influenced by mesenchymally derived fibronectin (149). The basal lamina lining the epithelial cells is continuous and apparently consists of components common to basement membranes in general, including type IV collagen, laminin, a heparan sulfate proteoglycan and fibronectin (150-153).

The dental basement membrane contains laminin; collagens type I, III and IV; heparan sulfate; hyaluronate and chondroitin 4- and 6-sulfate (150,152-155). Laurie, et al. (155) proposed that type IV

collagen, laminin, heparan sulfate proteoglycan and fibronectin form an integrated complex that comprises the basal lamina and its extensions. Collagen type IV, laminin and hyaluronate are derived from epithelium (146,147); collagen type I, type I trimer and type III (156,157) and chondroitin 4- and 6-sulfate (154) are from mesenchyme. Fibronectin might be synthesized by both tissues (149,152,153). Changes in the basement membrane occur during odontogenesis and odontoblast differentiation. Collagen type III disappears during terminal odontoblast differentiation, while fibronectin is seen only at the apical pole of odontoblasts (158).

It was suggested as early as 1934 that the dental epithelium plays a role in odontoblast terminal differentiation (159). Further work has established conclusively that developmental processes within the developing tooth are controlled by sequential and reciprocal epithelial-mesenchymal interactions (142,160-163). The action of the IEE on odontoblasts may be mediated by the intervening basement membrane (164). Control of IEE cellular activity by cells of the dental papilla may also be mediated by the basement membrane (144).

Koch (165) showed that heterotypic cell associations were required for the terminal differentiation of odontoblasts and ameloblasts. Later work demonstrated that odontoblast differentiation was time-dependent on the IEE (166). When the enamel organ and the dental papilla were separated by trypsin digestion to remove the basement membrane, reassociation was accompanied by redeposition of the basement membrane (167,168); redeposition of the basement membrane was a prerequisite for terminal differentiation of odontoblasts. EDTA dissociation of embryonic tooth organs separated the enamel organ from the dental papilla but

left an intact basement membrane in association with the papilla; in such cultures, post-mitotic odontoblasts polarized and secreted predentin (148,169).

The phenotype of the terminally differentiated odontoblast has not been completely defined. Prior to such differentiation, cells of the dental papilla synthesize collagen (type I, type I trimer and type III), glycosaminoglycans (GAGs; e.g., hyaluronate, heparan sulfate, chondroitin 4- and 6-sulfate, dermatan sulfate and keratan sulfate) and glycoproteins (e.g., fibronectin) (152-154,156,157,170,171). Odontoblast terminal differentiation lasts several hours and includes the following successive steps (127): (1) Odontoblasts withdraw from the cell cycle, become post-mitotic and polarize. The cells become larger with the nucleus assuming an eccentric basal location. Rough endoplasmic reticula elongate and become parallel with the long axis of the cell. (2) Cells synthesize and secrete predentin. An intact cytoskeleton is required both for polarization and for secretion; both functions are inhibited by colchicine and by cytochalasin B (172). An apical accumulation of the intermediate filament vimentin has been seen during polarization (173). Terminal differentiation of odontoblasts is marked by biosynthetic changes including increased synthesis of collagen type I and type I trimer with inhibition of type III synthesis (157,158), synthesis of dentin Gla-containing proteins (117,174) and the probable synthesis of fibronectin and other glycoproteins and GAGs (153,154,158).

The exact mechanisms of the control of odontoblast differentiation remain unclear, but it seems likely that terminal differentiation is controlled by an alternating flow of information between epithelial (IEE) and ectomesenchymal (dental papilla) cells (127,134,160,162).

Hypotheses for the transmission of these inductive signals include: (1) extracellular diffusion of molecules, (2) extracellular matrix-mediated interactions, (3) cell surface-mediated interactions of complementary structure, and (4) cell junction-mediated interactions (134). The basal lamina may contain inductive messages which are recognized by mesenchymal cells (175).

Differentiation of Ameloblasts

Ameloblasts terminally differentiate 24-36 hours after functional differentiation of odontoblasts (134). Cells withdraw from the cell cycle and polarize. This differentiation, as with odontoblasts, requires the integrity of the cytoskeleton (172,176), and the presence of predentin is an absolute prerequisite (177). During the terminal differentiation of ameloblasts, the basal lamina progressively disappears yielding direct heterotypic cell contact between odontoblast processes and pre-ameloblasts (163,178). In ameloblasts prekeratin and actin accumulated in the apical region, while uniform cellular distribution was present in pre-ameloblasts (173). Several proteins including enamelins (Mr = 72,000) and amelogenins (Mr = 25,000-30,000) serve as phenotypic markers (179,180).

Ameloblasts secrete an organic, water-rich enamel matrix which, as developing enamel, is 25-50% mineral and 20-30% matrix protein (181). These proteins, as mentioned above, are mainly amelogenins (90%, Pro-, Gln-, Leu- and His-rich, poorly phosphorylated or glycosylated and hydrophobic) and enamelins (10%, Ser-, Asp-, Glu- and Gly-rich, highly phosphorylated and glycosylated and acidic) (182). During the process of enamel maturation, protein content is decreased to approximately 0.3% in highly calcified enamel (183,184). This protein reduction is highly

weighted toward loss of amelogenins (182,184-186). Post-secretory ameloblasts are though to be responsible for this loss of organic material (187), although secretory ameloblasts may be involved in the removal of enamel matrix protein as well (183).

Dentin

Formation

Terminally differentiated odontoblasts secrete and elaborate a predentin matrix. At a site away from the odontoblast layer and termed the mineralization front (or the prendentin-dentin junction), the predentin matrix mineralizes to form mature dentin. With continue synthesis the odontoblasts migrate apically away from the thickening dentin, but they leave behind a cytoplasmic extension around which the dentin forms to create a dentinal tubule. The extent to which the process extends through this tubule into the dentin has been the subject of considerable research but still remains controversial (188). Many investigators have claimed that the process extends only partially into the dentin, although others have stated that the process completely traverses the width of the dentin to the dentinoenamel junction (for complete review, see Holland [188]).

Dentin Matrix

The composition of dentin matrix appears to be largely determined by the phenotype of the functional odontoblasts, although alteration of the matrix extracellularly seems likely (189). The major constituent of the organic matrix of both dentin and bone is collagen, comprising approximately 90% of the protein in the matrix. The rest consists of non-collagenous protein, lipid and proteoglycans (PGs).

Collagen

The identification of dentin collagen has been a difficult task because of its extreme insolubility. The most fruitful approach has proved to be analysis of peptides produced by CNBr cleavage in 70% formic acid of totally demineralized dentin (190,191). Dentin collagen consists primarily of type I collagen (190-192), although type I trimer (193-195) and type V (195) are present. The profile of predentin collagen is identical to that of dentin (196).

Non-collagenous Proteins

Since the organic matrix of both bone and dentin consists mainly of collagen, as described above, most early research focused on these proteins. However, much of this emphasis has been shifted to the other protein components, the NCPs of mineralized tissues. These NCPs are all anionic in character. For ease of discussion one can speak of five major classes of dentin NCPs (197).

Phosphoproteins - Phosphoproteins are the most abundant of the dentin NCPs (58% [w/w] in rat incisor [8]). The major phosphoprotein of bovine dentin, initially extracted during EDTA demineralization (198), has been termed phosphophoryn (199). This protein is distinguished by its unusually high content of serine and aspartate (545 and 452 of 1130 residues, respectively) (200). Approximately 95% of the serine residues are phosphorylated, giving the protein a tremendously high anionic charge density (201). Since at physiologic pH 80% of the phosphates will be doubly charged (9), phosphophoryn is extremely acidic (pI = 1.1 [rat] [202]), with an Mr = 155,000 for the bovine species (200). This highly phosphorylated protein is the most abundant and most thoroughly studied, although in many species several dentin phosphoproteins exist

(203). For example, rat incisor dentin contains two distinct, highly phosphorylated phosphoproteins and a third, less completely phosphorylated one (8,199). Amino acid analysis of this third protein reveals less aspartate and serine, more glutamate and glycine and some amino acid residues not present in the highly phosphorylated species (203). For the above reason, other investigators have designated phosphoproteins as having a high, intermediate or low degree of phosphorylation (203). Virtually all the organic phosphate exists as phosphoserine (204), although phosphothreonine is present in bone phosphoprotein (205).

Phosphoproteins strongly bind calcium. Bovine phosphophoryn binds Ca^{2+} with a binding constant of 3.6×10^4/moles (9), and, at high concentrations of phosphophoryn (~0.45 mg/ml), apparently one Ca^{2+} is bound for every aspartyl and phosphoseryl residue (201). At a reduced concentration (~0.05 mg/ml), only one third that amount of Ca^{2+} is bound (201). Bovine phosphophoryn also strongly binds to HA crystals (206).

Finally, a second set of phosphoproteins exists in dentin. In contrast to the above proteins, which were extracted during demineralization, this second group of proteins could be extracted only after degradation of the insoluble collagen matrix (7). This association and further data led to the initial suggestion that these phosphoproteins were covalently bound to collagen (207,208). The implications of such an association are discussed later in this manuscript.

Proteoglycans - PGs are large macromolecules composed of a protein core to which are linked mainly GAG side chains of repeating disaccharide units. These GAG chains are often sulfated and present a

high density of anionic charges (209,210). Of dentin GAGs, chondroitin-4-sulfate (C-4-S) and chondroitin-6-sulfate (C-6-S) predominate (211), although hyaluronate, dermatan sulfate (211) and heparan sulfate (212) are also present. PGs of dentin and predentin were studied separately (213). C-4-S was seen in dentin only, while both C-4-S and C-6-S were present in predentin (ratio 10:1, respectively). Dentin PGs are probably similar to those from bone (consisting of an Mr = 38,000 protein core and 1-2 GAG chains); the major point is that both species are quite different from cartilage PGs (210,214,215).

Gla-containing Proteins - Gla-containing proteins are abundant in dentin, comprising about 5% of all NCPs in rat dentin (8,115). These proteins can be separated into four fractions (termed γ_1-γ_4) by anion-exchange chromatography (116). Relatively few studies have been published regarding the dentin Gla-containing proteins in comparison to those of bone. However, considering the high degree of homology between the Gla-containing proteins of dentin and of bone (116), it seems reasonable to consider the chemistry of the dentin-derived proteins as being similar to that of the bone-derived material (189). The dentin Gla-containing proteins are further discussed elsewhere in this manuscript (117).

Glycoproteins - The majority of the other dentin NCPs are acidic glycoproteins, but relatively little is known about them (189). Some glycoproteins secreted by odontoblasts seem to be incorporated rapidly into mineralizing dentin and are absent from predentin (5). One Mr = 95,000 glycoprotein isolated from rat dentin, originally termed 95K, is a major dentin NCP and has been described in some detail (216). This protein is rich in aspartate, serine, glutamate and glycine and contains

34% (w/w) carbohydrate predominantly as glucosamine, galactosamine, mannose, galactose and sialic acid.

Plasma Proteins - Plasma proteins, including albumin, transferrin, IgG, IgM and IgA, have been detected in dentin (217). Albumin is released only after demineralization (8), indicating a strong affinity for the mineral component. α2HS-Glycoprotein may also be present (218). Debate continues as to whether any of these components are actually synthesized by odontoblasts or are instead just entrapped in the mineralizing dentin matrix.

Lipids

Lipids in dentin have been studied by many investigators and comprise approximately 0.2-0.3% of the dry weight or 1.7% of the total organic components (219). Total lipids are present in much greater quantities in predentin than in dentin (220). The lipid profile of dentin is generally similar to that of other tissues, composed mainly (95%) of phospholipids, cholesterol, cholesterol esters and triacylglycerols, with the remainder as free fatty acids, mono- and diacylglycerols (219,220). Several acidic phospholipids were found to be tightly mineral bound and could not be extracted until the dentin was completely demineralized (221-223). Much of the lipid that is present may exist in membrane-enclosed bodies, termed matrix vesicles, detected ultramicroscopically in dentin (224-227). In general, however, dentin lipids have been only poorly characterized and much work remains to analyze them more completely.

Mineralization

Dentin is a mineralized connective tissue which is first synthesized as an organic matrix that subsequently mineralizes. Mature dentin

is approximately 70% (w/w) inorganic mineral, 18% (w/w) organic material and 12% (w/w) water (228). The inorganic phase is chiefly calcium and phosphate existing as HA. The first dentin to be formed is the mantle dentin, a layer of variable thickness (approximately 2-10 μm) which is located peripherally, eventually to lie adjacent to enamel (229). It contains coarse fibrils grouped into fibers (230). The remainder and the bulk of the dentin is termed circumpulpal dentin; its fine collagen fibrils generally lie within the plane of the developing surface (231). Mantle dentin seems not to be as completely mineralized as circumpulpal dentin (232,233).

The formation of mineralized tissue has been the subject of much research for a great many years, but the exact nature of the biochemical events that lead to this specific deposition of mineral remains obscure. Many hypotheses have been proposed, and the following molecules and macromolecules have been suggested as having a role in mineralization: (1) collagen (234); (2) phosphoproteins (199); (3) alkaline phosphatase (235); (4) Gla-containing proteins (14); (5) glycoproteins (236); (6) PGs (189); and (7) lipids (as isolated molecules or as components of matrix vesicles) (227,237,238).

Collagen

Since the collagens of bone, dentin and cementum are chiefly type I, it has been presumed that the molecular structure of the collagen is important in determining whether that tissue can mineralize (239). The presence of other collagen types in other tissues may prevent their mineralization. Collagen cross-linking may be involved in this regulation. It has been proposed (240) that multifunctional cross-links in non-mineralized collagen hold the molecules more tightly, preventing the

entrance of ions and the formation of HA crystallites. On the other hand, once bone collagen is mineralized, the presence of mineral does not allow for the formation of these cross-links (240).

Mineral appears to form in a specific spatial order around the collagen fibrils. The majority of the apatite crystals seem to be aligned with the same axial periodicity as the collagen molecules and in the collagen hole regions (241-243). It appears that the fibrillar packing of collagens in mineralized tissues is more loosely arranged, and this loose arrangement may be necessary to allow for mineralization (244). Collagen may also act to limit the amount of mineral that is deposited (242).

Phosphoproteins

Phosphoproteins may play a role in the mineralization of organic matrices by providing a nucleation site for apatite formation (199). Indeed, at lower concentrations phosphoproteins will induce formation of HA in supersaturated calcium-phosphate solutions in which would otherwise form amorphous calcium-phosphate (10). At higher concentrations, however, the conversion to HA of amorphous calcium-phosphate was inhibited (245). This inhibition, though, may have resulted simply from the reduction of free ion concentrations to a point below the solubility product (210). It has been proposed that some dentin phosphoproteins may be covalently bound to collagen and perhaps serve as a bridge linking mineral to matrix (206,246-248). Such an interaction may serve to increase calcium-binding affinity of the collagen surface and may nucleate growth of apatite crystals along their C axis (201). This concept of covalent linkage has been disputed (249) and is open to various interpretations. It is of interest to note, however, that

autoradiographic data have suggested that a phosphoprotein synthesized by odontoblasts is carried through predentin and is secreted directly at the mineralization front where it may participate in mineral deposition (4). Biosynthetically labeled rat incisor phosphoproteins reach the mineralization front within 15 minutes of labeling (250). Clearly, more work is necessary to elucidate the role of phosphoproteins in the mineralization process.

Alkaline Phosphatase

Ever since the early work of Robison (235) and Kay (251), skeletal alkaline phosphatase has been suggested to play a role in mineralization, perhaps by hydrolyzing phosphate esters and increasing local phosphate concentrations. Alkaline phosphatase is an enzymatic marker of extracellular matrix vesicles (252), claimed by some investigators to be the sites of initial dentin mineralization (227,253). Alkaline phosphatase in matrix vesicles is a glycoprotein with a high affinity for Ca^{2+} (254) and may transport inorganic phosphate into these vesicles (255). In addition, an inorganic pyrophosphatase exists in developing dentin (256).

Gla-containing Proteins

The possible roles suggested for Gla-containing proteins in mineralization have been discussed above and elsewhere in this manuscript (117).

Glycoproteins

As described above and in a manner similar to that for phosphoproteins, data from autoradiographic studies have suggested that glycoproteins synthesized by odontoblasts are transported through predentin and

are secreted directly at the mineralization front (5). Presumably, labeled glycoproteins somehow may participate in mineral deposition.

Proteoglycans

It is generally considered that PGs are present in bone and dentin to act as regulators of mineralization (189,257-259). Bovine nasal PGs in a metastable calcium-phosphate solution delay or prevent HA formation in a dose-dependent fashion (260). PGs also inhibit the transformation of amorphous calcium-phosphate to HA (260). The large, highly anionic PG aggregates (and monomers to a lesser extent) occupy much tissue space and presumably bind water to reduce significantly the rate of diffusion of ions (e.g., Ca^{2+} and HPO_4^{2-}) through solution. It seems that PGs may act as a steric hindrance for mineralization (261).

A different function has been proposed for PGs based on their ability to interact with collagen and control the organization of the tissue (262-265). PGs may accelerate fiber formation initially and retard formation after initial fibrillogenesis (265). PGs may stabilize collagen fibril assemblies in vivo (266).

Lastly, PG metabolism may be important for dentin mineralization. The concentration of sulfur, presumably in PGs, is lower in rat incisor dentin than in predentin (267). This decrease occurs just after the mineralization front (267). Evidence exists for a loss of PGs during mineralization in both dentin (258) and bone (268,269). There may even be two pools of PGs involved in dentinogenesis, one incorporated quickly into mineralized dentin and the other secreted into predentin and then at least partially metabolized (213,270).

Osteonectin

Many investigators have searched for a biological "bridge" that could link bone matrix collagen to mineral. Osteonectin, a phosphorylated and glycosylated (11) bone NCP (Mr = 32,000-40,000 [11,271]) has been promoted as possibly fulfilling this role (12). In vitro data support this view. Osteonectin binds specifically both to HA (11) and to denatured collagen (11,12). Preliminary data suggest that it binds HA with greater affinity than do several other NCPs (e.g., BGP) (272). Since osteonectin is produced by osteoblasts (21,273), the above data suggest that osteonectin may be responsible for collagen mineralization in bone (13). The observation that bone matrix from calves with a type of osteogenesis imperfecta was deficient in osteonectin (less than 5% of control) also points to a role for osteonectin in mineralization (274). However, an osteonectin-like protein is also synthesized by some fibroblasts (275). Final elucidation of the role of this protein depends on further work with more specific probes.

Lipids

Lipids have been implicated in mineralization in several ways. A frequently cited role focuses on the ability of certain anionic phospholipids to bind Ca^{2+} with moderate affinity and specificity (276,277). Binding of Ca^{2+} in the presence of inorganic phosphate (Pi) leads to the formation of phospholipid-Ca-Pi complexes (276). These complexes have been isolated from mineralizing (but not from non-mineralizing) tissues (237,278,279). Such complexes can nucleate HA from metastable calcium-phosphate solutions (237). Proteolipids, present as membrane components

of most cells, have also been implicated in mineralization. Some, but not all, proteolipids can induce in vitro mineralization (238).

Finally, any discussion of lipids must consider matrix vesicles. Matrix vesicles are extracellular bodies of cellular origin consisting of an amorphous substance surrounded by a membrane (280). Matrix vesicles are present in dentin, readily apparent in mantle (225,227,281) but not circumpulpal (224,226) dentin. These findings are consistent with the idea that matrix vesicles are needed only at the site of initial dentin mineralization, but they also may indicate simply a greater difficulty in isolating such vesicles from the more highly mineralized circumpulpal dentin. Many investigators have proposed that matrix vesicles are the site of initial mineralization (for review, see Bonucci [280]). The mechanism by which these structures may act as foci for initial mineral nucleation remains a matter of controversy. Alkaline phosphatase activity may be involved (282). Also implicated within matrix vesicles are proteolipids and phospholipid-Ca-Pi complexes as described above.

HYPOTHESIS AND OBJECTIVES

Gla-containing proteins are abundant in the organic matrix of bone and dentin. Although such proteins have been the subject of extensive investigation, their ultimate biological function(s) remains obscure.

The biosynthesis of Gla-containing proteins of mineralized tissues also is a matter of controversy. One question involves the developmental appearance of Gla-containing proteins relative to the onset of mineralization. Since Gla proteins are synthesized by both osteoblasts and odontoblasts, the question could alternatively be asked whether the presence of mineral is necessary in order to see biosynthesis of Gla proteins. This question has been studied regarding bone, but the answer remains equivocal. Reports for the appearance of BGP both before (or perhaps concurrently with) and after first bone mineral have been discussed earlier in this manuscript. To date, however, this question has not been addressed concerning odontoblasts and dentin. Thus, the hypothesis tested in this report was that odontoblasts synthesize dentin Gla proteins in developing teeth and that this biosynthesis is evident prior to the appearance of mineralized dentin.

In order to study this hypothesis, two goals had to be reached. The first involved the model system. An <u>in vitro</u> model of mineralization involving odontoblasts had to be developed. The developing rat molar was chosen as the model for this study for two reasons: (1) Odontoblasts from rat have been shown to synthesize dentin Gla proteins;

and (2) the molar organ is a well-defined model. In it are a variety of cell types, and the development of the molar has been thoroughly studied. Histogenesis, morphogenesis and tooth development proceed in an orderly and sequential fashion, the timing of which is fairly well understood. In addition the cells are arranged so that one knows precisely the developmental relationships involved. The goal was to use the molar to develop an in vitro model of mineralization and dentinogenesis.

The second goal of this research was to use this characterized model to study the biosynthesis of the dentin Gla proteins in relation to mineralization and for studies regarding their possible biological significance.

Following this section are two manuscript reprints. The first discusses the fat soluble vitamin D and describes an interesting relationship between vitamin D and BGP. The second outlines most of the experimental work with the molar organ and dentin Gla protein biosynthesis. Following the second reprint will be a series of appendices describing the molar organ culture system in detail and presenting further results obtained from this research. Also following the reprints will be a closing discussion and suggestions for future research.

REVIEW ARTICLE: VITAMIN D AND SKELETAL TISSUES

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ABSTRACT

It is now accepted that vitamin D is an integral part of a complex endocrine system, one with far-reaching implications in mineral metabolism. Reviews of the sources, functions and metabolism of vitamin D, as currently understood, are presented as a prelude to discussions of the role of vitamin D in calcium and phosphorous homeostasis and possible specific roles for vitamin D in mineralized tissues. Data describing a possible regulatory function for vitamin D in bone and bone protein metabolism are presented. Some of the controversy which presently exists regarding the biochemical mechanism of the action of this vitamin is discussed. Finally, the possible relationship of vitamin D and disorders of skeletal tissues is described.

Vitamin D has long been known as the antirachitic vitamin, but the exact biochemical nature of its action has remained obscure (1). Rickets as a disease entity was seen as early as the mid-1600's and appeared with greatly increased frequency in both North America and Europe as much of the population moved indoors during the industrial revolution (2). This increased frequency correlated with an increase in atmospheric smoke and pollution and a reduced exposure to sunlight. Indeed, it was discovered that much of this human rickets could be cured with exposure to ultraviolet light (3).

Vitamin D_2, ergocalciferol, was isolated and identified in the early 1930's (4,5), and vitamin D_3, cholecalciferol, was identified a short time later (6). Since that time, and especially in the last 20 years, much research has been directed toward the elucidation of the actions of vitamin D (7). Along with the great advances that have been made in the understanding of the vitamin D system, much controversy has also resulted from the numerous conflicting reports and results that have been published (8). The purpose of this report is to review what is currently known about vitamin D and to present some of the data that have led to the suggestion of possible specific roles for vitamin D in bone metabolism.

SOURCES OF VITAMIN D

Vitamin D itself is not found in many foods; its major sources are fish liver oils, with much smaller amounts in egg yolks and glandular meats (9,10). Apparently, one reason for this lack of distribution among foods is that vitamin D is produced in the skin. 7-Dehydrocholesterol, present in the skin in fairly large quantities as an intermediate in the biosynthesis of cholesterol, is converted to previtamin D_3 upon

irradiation with ultraviolet light (11,12). To date, no enzyme or protein has been shown to be necessary for this conversion, and no side reaction products have been seen in the skin (11,13). Previtamin D_3 is in a thermally dependent equilibrium with vitamin D_3, an equilibration which occurs slowly at 37°C (13). This provides a slow influx of vitamin D_3 into the body (1). The plasma transport vitamin D binding protein (DBP) binds to vitamin D_3 but not to previtamin D_3, so apparently this is the level of selection for vitamin D_3 (13). DBP was also found to bind actin, a ubiquitous and highly conserved intracellular protein, but the significance of this is not yet understood (14). Thus, vitamin D actually should be considered a vitamin only in the absence of ultraviolet light. The structures and numbering system for both previtamin D_3 and vitamin D_3, along with the mechanism of their formation from 7-dehydrocholesterol, are illustrated in Fig. 1.

ACTIONS OF VITAMIN D

The classical description of vitamin D deficiency is the childhood disease rickets in which there is a failure to deposit calcium-phosphate mineral crystals in the form of hydroxyapatite onto the organic matrix of bone (Fig. 2). As the bone grows, the weight of the growing child causes the bending and limb deformation that is characteristic of rickets. In the adult, since there is constant turnover and remodeling of bone, new bone matrix synthesized by osteoblasts fails to mineralize. This disease, called osteomalacia or adult rickets (15), leads to weakness in the bone and can cause severe bone pain.

It appears that the basic defect in bone mineralization during vitamin D deficiency is an insufficient serum concentration of calcium and phosphorous. In vitamin D deficiency, the blood is undersaturated

Fig. 1 - Conversion of 7-dehydrocholesterol to vitamin D_3 and numbering system. UV indicates ultraviolet light; kt indicates thermal equilibration.

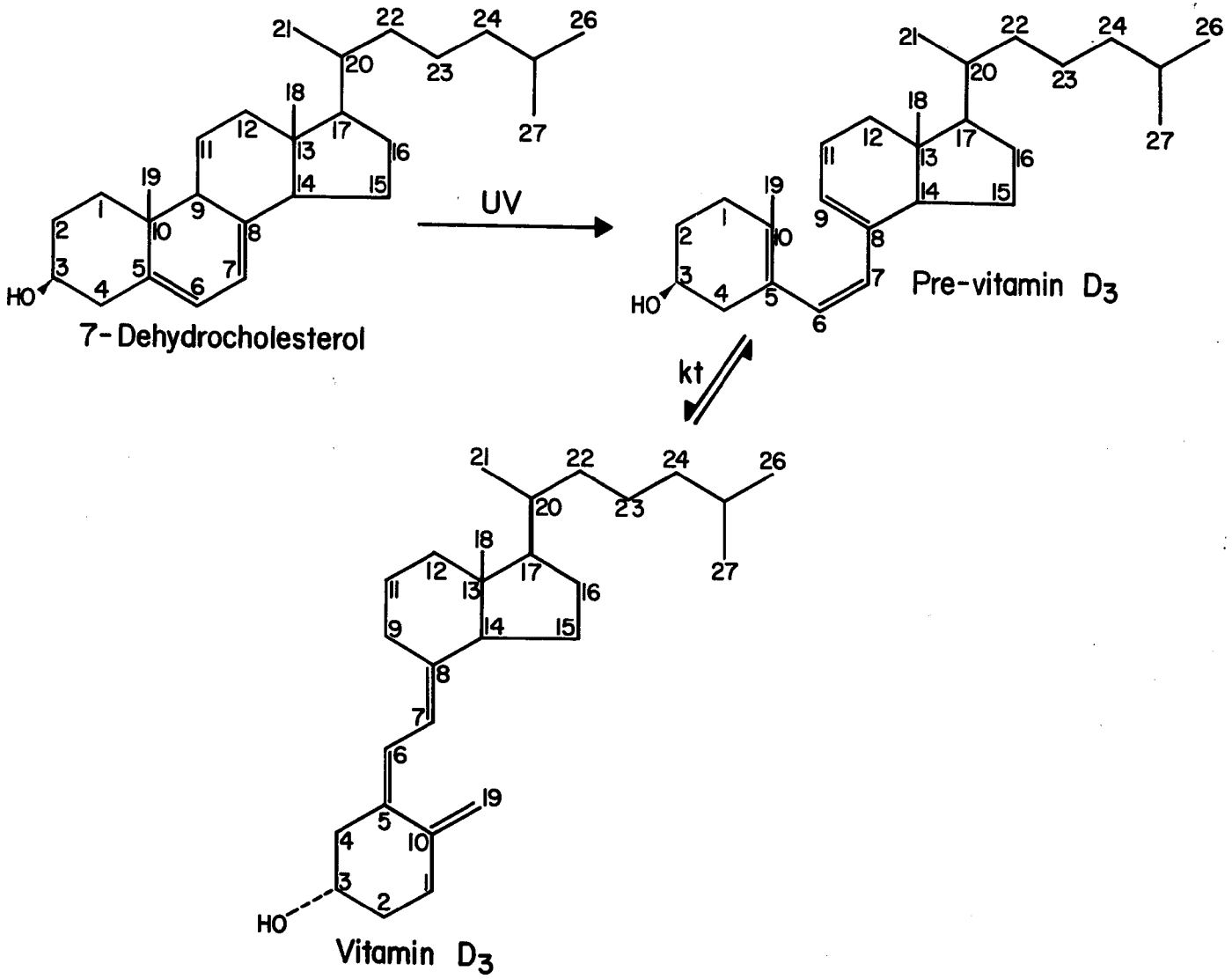
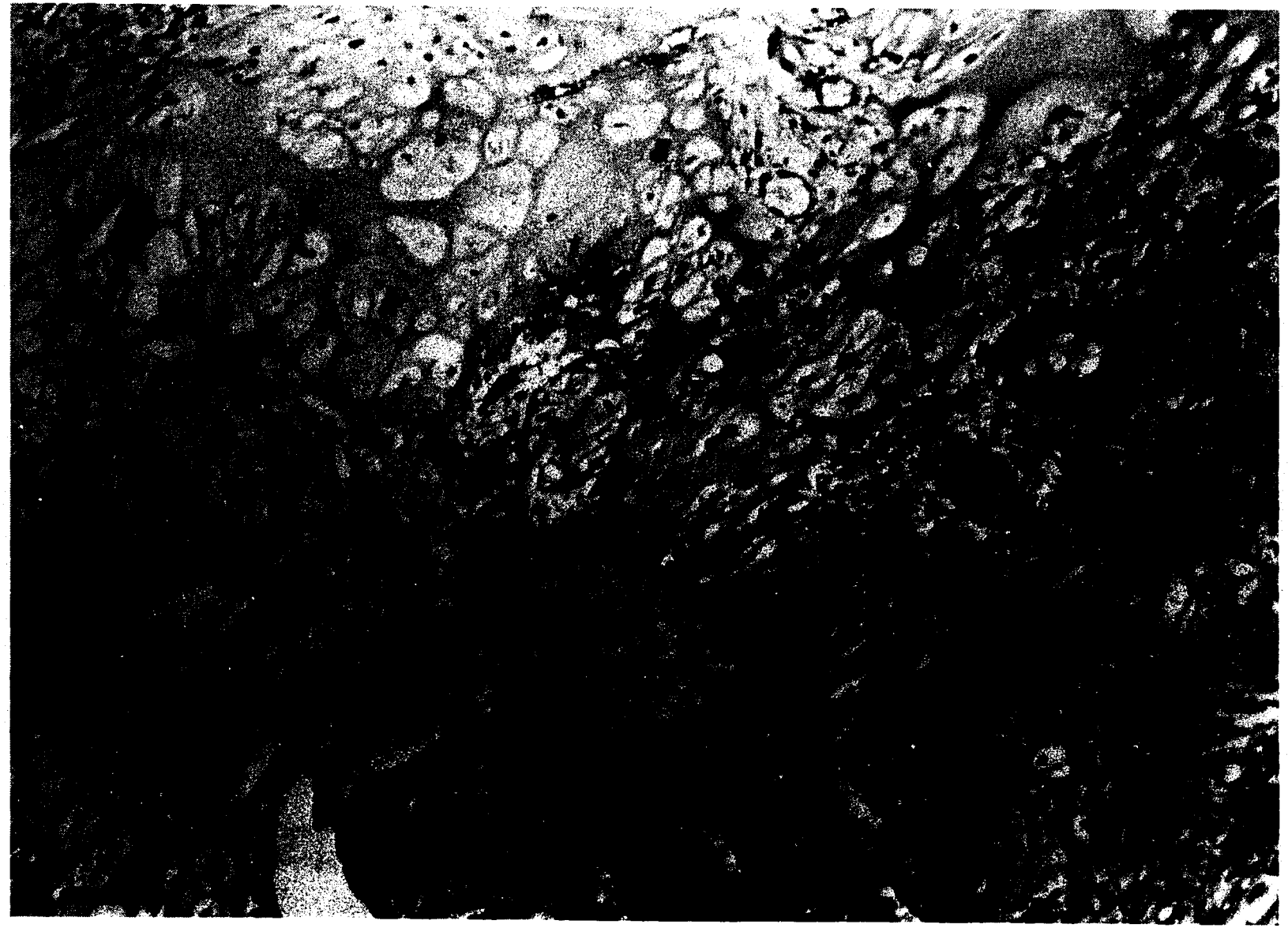

UV
kt
7-Dehydrocholesterol
Pre-vitamin D3
Vitamin D3

Fig. 2 - Photomicrograph of costochondral junction from patient with rachitic rosary. Note abnormal vascularization of cartilage with irregularly arranged osteoid (arrow) (H&E, x 196.5. Bar = 84.8 μm).



with both these ions and cannot facilitate their transfer to the extracellular fluid space in order to support normal mineralization (16). So the fundamental role of vitamin D is to raise the serum concentrations of calcium and phosphorus to supersaturating levels (9).

To accomplish this function, vitamin D acts on two, or perhaps three, target organs (10,17). It appears that the major site of action is the small intestine where vitamin D stimulates the transfer of calcium from the intestinal lumen to the plasma (18). At this same site it also stimulates the transfer of phosphate to the plasma by a separate but largely unknown mechanism (19).

In the skeleton vitamin D facilitates the transport of calcium from the bone fluid compartment to the extracellular fluid compartment (10,20). This requires the presence of both vitamin D and parathyroid hormone (PTH) (21). This system is functional mainly when intestinal absorption of calcium and phosphate is insufficient to meet demands (2). Vitamin D is also known to play an important role in bone remodeling (22). Incubation of embryonic bone with vitamin D and its metabolites *in vitro* causes a marked resorption of that bone (23,24). Resorption of bone appears to be tightly coupled to formation of new bone for replacement both *in vivo* (22,25) and *in vitro* (26).

Finally, in the kidney, vitamin D is believed to act together with PTH to stimulate the final reabsorption of calcium in the distal tubule (10,27). It is known that 99% of the filtered calcium is reabsorbed even in the absence of both PTH and vitamin D. The remaining 1% is controlled by PTH (28) and perhaps by vitamin D (29). However, since approximately 7-10 g of calcium are filtered in man per day, if vitamin D does indeed influence the reabsorption of the remaining 1%, 1% of that

7-10 g could represent a significant amount of calcium (30). Further experiments are required to resolve this question.

METABOLISM OF VITAMIN D (Fig. 3)

The most dramatic breakthrough in the understanding of vitamin D occurred in the late 1960's with the realization that vitamin D must be metabolically altered before it is biologically active (31,32). The chief clue for this alteration was the discovery that a 12-h lag period is required after intravenous injection of vitamin D_3 before a target-organ response is observed (32). This concept of metabolic alteration became firmly established with the identification of the major circulating form of vitamin D, 25-hydroxyvitamin D_3[1] [$25(OH)D_3$] (34), a compound shown to be more active and more rapidly acting than parent vitamin D_3 (35).

This discovery led to an interest in the further metabolism of vitamin D; such metabolic studies were made possible by the synthesis of radiolabeled $25(OH)D_3$ (36). Radiolabeled $25(OH)D_3$ is rapidly metabolized when injected intravenously (37). Holick et al. (38) reported the isolation and identification of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] from the intestine of vitamin D-deficient chicks given a dose of radiolabeled vitamin D. The configuration of the 1-hydroxyl group was shown to be α (39). Vitamin D_3 is first converted to $25(OH)D_3$ in the liver and then to $1,25(OH)_2D_3$ in the kidney before it is functional

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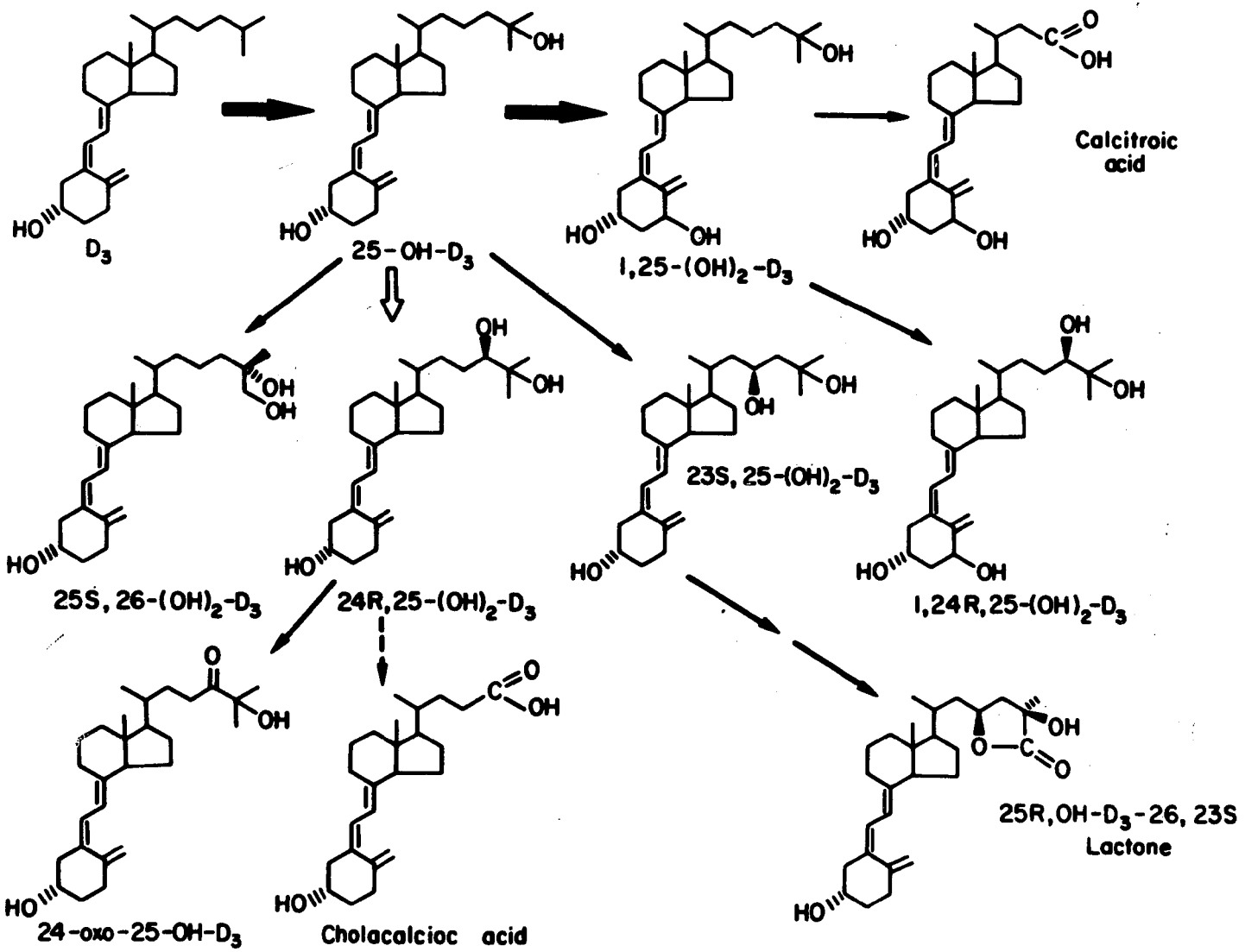
[1]The International Union of Pure and Applied Chemistry - International Union of Biochemistry (IUPAC-IUB) (33) has recommended that the following trivial names be adopted: vitamin D_3, calciol or cholecalciferol; 25-hydroxyvitamin D_3, calcidiol; 1α,25-dihydroxyvitamin D_3, calcitriol; 24R,25-dihydroxyvitamin D_3, (24)-hydroxycalcidiol; 1α,24R,25-trihydroxyvitamin D_3, calcitetrol.

Fig. 3. Metabolism of vitamin D_3 to its major identified metabolites. Please note other metabolites of minor concentration isolated from in vivo sources following large doses of vitamin D, or from in vitro sources without demonstration of in vivo existence, are not illustrated here because it is uncertain whether they are of significance in vivo. (Reprinted from DeLuca HF, Schnoes HK, Ann Rev Biochem 1983: 52: 411, with permission from Annual Reviews Inc., publishers.)


D3
25-OH-D3
1,25-(OH)2-D3
Calcitroic acid
25S, 26-(OH)2-D3
24R,25-(OH)2-D3
23S, 25-(OH)2-D3
1,24R,25-(OH)2-D3
24-oxo-25-OH-D3
Cholacalcioc acid
25R,OH-D3-26, 23S Lactone

(40). The liver is the major site of $25(OH)D_3$ production (41,42); the kidney is necessary for the production of $1,25(OH)_2D_3$ (43). Hepatectomy markedly reduces the former conversion (42), while nephrectomy prevents the latter (44). Also in nephrectomized animals, $25(OH)D_3$ in physiologic amounts cannot stimulate intestinal calcium tranport, while $1,25(OH)_2D_3$ will stimulate this transport independently of the kidneys (45,46). A similar experiment demonstrated that $1,25(OH)_2D_3$ is the active form necessary for the mobilization of calcium from bone (47).

The conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ occurs in the kidney; the targets for the action of $1,25(OH)_2D_3$ are at sites away from its synthesis and reached via blood transport. Thus, it is clear that $1,25(OH)_2D_3$, having satisfied all the appropriate criteria, must be considered as a hormone (40). This realization led to an entirely new view of the calcium and phosphorous homeostatic mechanism - the vitamin D endocrine system.

The liver 25-hydroxylation of vitamin D is accomplished by a microsomal two-component mixed-function monooxygenase system (48,49). One component is a flavoprotein and the second is a cytochrome P-450. Endogenous reduced nicotinamide adenine dinucleotide phosphate (NADPH) reduces the flavoprotein which in turn reduces the cytochrome P-450. The cytochrome P-450 then reduces molecular oxygen to water and the 25-hydroxyl which is substituted on vitamin D. In addition to the microsomal system, liver mitochondria can also 25-hydroxylate vitamin D (50). However, the Michaelis constant for the mitochondrial reaction is on the order of 10^{-6} M (30,50), while it is on the order 10^{-8} M for the microsomal system (48). A mitochondrial enzyme will also 25-hydroxylate cholesterol, but the microsomal one will not (7). However, this

mitochondrial hydroxylase appears to be a different enzyme than the one active on vitamin D_3 (50). There also appear to be sites for 25-hydroxylation in intestine and in kidney, but the total extrahepatic activity is minimal, and the liver can be considered the major site for the production of $25(OH)D_3$ (42,51,52).

$25(OH)D_3$ is transported from the liver via DBP to the kidney where it is a substrate for the enzyme system $25(OH)D_3$-1α-hydroxylase. $25(OH)D_3$ is 1α-hydroxylated solely in the mitchondria by a three-component mixed-function monooxygenase (53-56). Once again, endogenous NADPH is the ultimate reductant, reducing a flavoprotein. This flavoprotein reduces an iron-sulfur protein complex termed renal ferredoxin, which subsequently reduces a cytochrome P-450. The cytochrome P-450 in turn reduces molecular oxygen to yield water and the product, $1\alpha,25(OH)_2D_3$. The components have been assembled into a soluble hydroxylase system, and reconstitution and kinetic studies with chick $25(OH)D_3$-1α-hydroxylase have shown that the mitochondrial cytochrome P-450 is the final oxidase of the system (55). Of the three components, the renal ferredoxin, an Fe_2S_2 species with an apparent molecular weight (Mr) of 11,800, was isolated and purified initially (57,58). The other two components, the flavoprotein, called NADPH-renal ferredoxin reductase (NADPH-renoredoxin reductase by the authors), and the cytochrome P-450, tentatively termed cytochrome $P\text{-}450_{D1\alpha}$, were isolated and purified to apparent homogeneity a short time later (59,60). The cytochrome $P\text{-}450_{D1\alpha}$ is specific for 1α-hydroxylation of $25(OH)D_3$ (60). This second hydroxylation reaction is the primary reason that vitamin D function should be considered as an endocrine system, since it is this reaction

that yields the compound which is generally considered to be the active hormonal form of vitamin D, $1,25(OH)_2D_3$ (2).

More recently, data have been presented describing extrarenal production of $1,25(OH)_2D_3$ both *in vivo* (61-63) and *in vitro* in bone (64,65) and in chick chorioallantoic cells (66). Clearly, the placenta is one extrarenal site of $25(OH)D_3$-1α-hydroxylation (67-69), but recent work using radiolabeled $25(OH)D_3$ of high specific activity, in which no radiolabeled $1,25(OH)D_3$ could be found in anephric, nonpregnant animals, raises a serious question as to whether these nonplacental, extrarenal sites of $1,25(OH)_2D_3$ production are of any biological importance *in vivo* under normal conditions (70).

Another major metabolite of vitamin D found in plasma, isolated and identified in the early 1970's, is 24,25-dihydroxyvitamin D_3 [$24,25-(OH)_2D_3$] (71). The configuration at carbon 24 was shown to be R (72). $24R,25(OH)_2D_3$, produced by kidney, intestine, cartilage and perhaps other tissues (73-75), cannot be considered to be a hormone. Additionally, the 24R-hydroxylase enzyme system, also a mixed-function monooxygenase (76), will function with $1,25(OH)_2D_3$ as a substrate as well as with $25(OH)D_3$ to yield 1,24R,25-trihydroxyvitamin D_3 [$1,24,25(OH)_3D_3$] (77). Data from *in vitro* studies with rat kidney preparations have shown that the 24R-hydroxylase is also a mitochondrial enzyme, dependent on NADPH as the ultimate reductant (78) and using a cytochrome P-450 which is distinct from that of $25(OH)D_3$-1α-hydroxylase (60). Vitamin D-deficient tissues show no 24-hydroxylase activity, but the enzyme is induced by several forms of vitamin D with $1,25(OH)_2D_3$ being the most active (79).

It has been suggested that 24R,25(OH)$_2$D$_3$ has unique functions in embryonic development and chick egg hatchability (80,81), in suppressing PTH secretion (82) and in bone mineralization (83-85), but these proposals have yet to be substantiated. There may even be high-affinity binding sites for 24R,25(OH)$_2$D$_3$ in bone (86), in epiphyseal cartilage cells (87) and in parathyroid gland (81). However, 24,24-difluoro-25(OH)D$_3$ has been prepared (88,89) and was found to bind to receptors in the same manner as 25(OH)D$_3$ (90). In addition, 24,24-difluoro-25(OH)D$_3$ was shown to exhibit as much biologic activity as 25(OH)D$_3$ in all functions including bone mineralization (90-93). Indeed, 24,24-difluoro-1,25(OH)$_2$D$_3$ was shown to have a potency 5-10 times that of 1,25(OH)$_2$D$_3$ in vivo (94). Since the C-F bond is stable and not subject to 24-hydroxylation, these data seem to refute the suggestion that 24R,25(OH)$_2$D$_3$ has special functions. It is entirely possible that 24-hydroxylation may represent the first step in deactivation, perhaps resulting in a water soluble C-24 carboxylic acid (95).

Two recent reports have addressed the question of 24,25(OH)$_2$D$_3$ and bone more closely. Dickson et al. (96) report that 1,25(OH)$_2$D$_3$ alone can prevent most of the manifestations of rickets, while 24,25(OH)$_2$D$_3$ has much lower antirachitic properties with minimal capabilites for healing rachitic lesions. The authors do suggest, however, that a combination of 1,25(OH)$_2$D$_3$ and 24,25(OH)$_2$D$_3$ promotes the mineralization of subperiosteal trabeculae and that 24,25(OH)$_2$D$_3$ may directly stimulate chondrocyte proliferation, since 24,25(OH)$_2$D$_3$ treatment caused a widening of the zone of proliferation in chick bone concomitantly with a disorganization of the columnar arrangement of cells. Goodman et al. (97) report that 24,25(OH)$_2$D$_3$ can promote the maturation and

mineralization of osteoid, can restore serum calcium to normal values and seems to possess antirachitic properties in azotemic, D-deficient rats. The authors suggest that $24,25(OH)_2D_3$ alone can correct several of the effects of vitamin D deficiency on bone in these animals. The results are open to question, however, since these rats had undergone a subtotal nephrectomy, raising the possibility that the formation of $1,24,25(OH)_3D_3$ in the remaining renal tissue has caused the observed effects. Indeed, the conversion of $24,25(OH)_2D_3$ to $1,24,25(OH)_3D_3$ is necessary for this metabolite to stimulate intestinal calcium absorption in the rat (98). Clearly, further efforts are required to clarify this question.

Additional metabolites of vitamin D have been discovered. When 26,27-[^{14}C]$25(OH)D_3$ (99) was given to vitamin D-deficient animals, 7% of the radioactivity appeared in expired CO_2 (100). When 26,27-[^{24}C]$1,25-(OH)_2D_3$ was given to vitamin D-deficient animals, 30% of the radioactivity appeared in expired CO_2 within 24 h following administration (100). These data suggest that side chain oxidation mainly uses $1,25(OH)_2D_3$ as a substrate, since nephrectomy eliminates this metabolic pathway. A water soluble metabolite has been identified as calcitroic acid (101), believed to be an inactivated form of $1,25(OH)_2D_3$ which is rapidly eliminated in the bile (2).

Another metabolite, $25(OH)D_3$-26,23-lactone, was detected by competitive binding with DBP (102). Initial experiments have shown it to be virtually devoid of biological activity on calcium transport in intestine or calcium mobilization from bone (30).

Numerous other more minor metabolites of vitamin D have been identified from both <u>in vivo</u> and <u>in vitro</u> sources [for complete review,

see DeLuca and Schnoes (103)]. It is unknown, however, whether these other minor species represent biologically significant molecules.

VITAMIN D REGULATION AND CALCIUM HOMEOSTASIS

Studies of the relationship of PTH and $1,25(OH)_2D_3$ functions have revealed the calcium homeostatic mechanism (104) (Fig. 4). In conditions of hypocalcemia, PTH is secreted and binds to receptors in kidney and bone but not in intestine (40). In the kidney, PTH inhibits phosphate reabsorption (104), stimulates the $25(OH)D_3$-1α-hydroxylase (105-107) and, theoretically with $1,25(OH)_2D_3$, stimulates the final reabsorption of calcium (27,108). The $1,25(OH)_2D_3$ produced in the kidney then advances to intestine, bone and other sites in the kidney (2). It stimulates the mobilization of calcium from bone (which requires the presence of PTH), stimulates intestinal calcium transport (independently of PTH) and apparently functions with PTH to stimulate renal calcium conservation as described earlier (27). As calcium concentrations rise, the change is sensed by the parathyroid gland and PTH is no longer released. $1,25(OH)_2D_3$ itself may also inhibit PTH secretion in a feedback mechanism independently of serum calcium (109). If calcium concentrations increase above normal, calcitonin is secreted from the parafollicular cells of the thyroid. This hormone inhibits calcium mobilization from bone (110), inhibits bone resorption (104,111) and has been shown to inhibit the bone resorption induced by $1,25(OH)_2D_3$ (112, 113), $25(OH)D_3$ (114) and PTH (114,115) <u>in vitro</u>, although this effect may be short-lived (114).

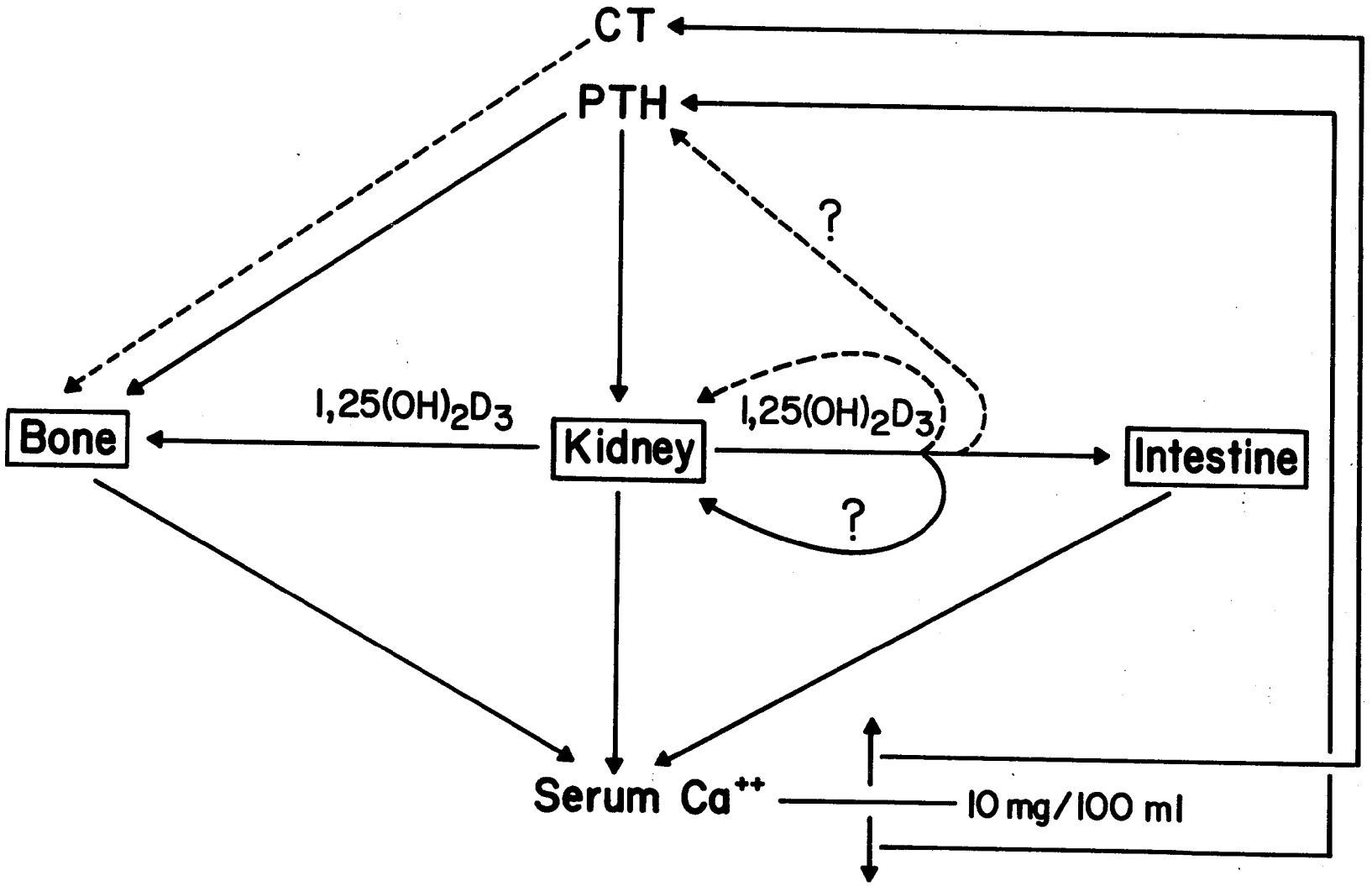
Production of $1,25(OH)_2D_3$ is closely regulated by serum calcium (116). At normal calcium concentration (10 mg/100 ml), both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ are produced in small amounts. During periods of

hypocalcemia, $1,25(OH)_2D_3$ production is increased while $24,25(OH)_2D_3$ production is inhibited (116,117). Conversely, under conditions of hypercalcemia, 24-hydroxylation is stimulated while 1-hydroxylation is suppressed (30). The ability of hypocalcemia to stimulate 1-hydroxylation of $25(OH)D_3$ is destroyed by parathyroidectomy (105). PTH given to parathyroidectomized animals will stimulate 1α-hydroxylation while inhibiting 24R-hydroxylation (105,118). Thus, the parathyroid gland, the primary calcium regulating organ, plays a major role in the regulation of $1,25(OH)_2D_3$ production (105).

The action of PTH in kidney cells may be mediated through cyclic adenosine 3'5'-monophosphate (cAMP) in a manner similar to other peptide hormones, with PTH perhaps stimulating adenylate cyclase activity to yield an increase in the intracellular concentration of cAMP (106,119). Both cAMP (120) and dibutyryl cAMP (121) will mimic the action of PTH and stimulate the $25(OH)D_3$-1α-hydroxylase in renal tubules <u>in vitro</u>, while cAMP will stimulate $1,25(OH)_2D_3$ production when administered to thyroparathyroidectomized, vitamin D-deficient rats <u>in vivo</u> (122). However, experiments to define the nature of PTH regulation of the renal $25(OH)D_3$-1α-hydroxylase with preparations isolated from kidney cells thus far have not been successful (123,124).

$1,25(OH)_2D_3$ itself regulates both $25(OH)D_3$-1α-hydroxylase and $25(OH)D_2$-24R-hydroxylase activity. $1,25(OH)_2D_3$ suppresses the 1-hydroxylase while stimulating the 24-hydroxylase (79,125,126). A nuclear mechanism for this response involving regulation at the transcriptional level appears likely since this regulatory activity of $1,25(OH)_2D_3$ is blocked by both actinomycin D and α-amanitin (i.e.,

Fig. 4. Schematic representation of calcium homeostatic mechanism involving parathyroid hormone (PTH), calcitonin (CT) and the vitamin D hormone, 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Hypocalcemia (serum [Ca^{++}] < 10 mg/100 ml) stimulates PTH release, which stimulates $1,25(OH)_2D_3$ production in kidney, renal calcium conservation and bone resorption. $1,25(OH)_2D_3$ stimulates intestinal calcium absorption, acts together with PTH to stimulate calcium mobilization from bone and perhaps joins with PTH to stimulate renal calcium conservation. $1,25(OH)_2D_3$ exerts feedback control on kidney and perhaps on parathyroid gland. Hypercalcemia (serum [Ca^{++}] > 10 mg/100 ml) stimulates CT release, inhibiting calcium mobilization from bone. (⟶) indicates stimulation; (--→) indicates inhibition.


CT
PTH
?
Bone
1,25(OH)2D3
Kidney
1,25(OH)2D3
Intestine
?
Serum Ca++
10 mg/100 ml

inhibitors of transcription) (125). These data also seem to support the idea that 24-hydroxylation leads to inactivation.

The metabolism of vitamin D is also regulated by serum phosphate (127). Under conditions of hypophosphatemia, the concentration of circulating $1,25(OH)_2D_3$ is markedly increased, while high phosphate, on the other hand, reduces plasma concentrations of this metabolite (117). It has been suggested that low blood phosphate stimulates $25(OH)D_3$-1α-hydroxylase activity directly (128), but this has not been verified. However, a recent report has shown that restriction of dietary phosphate causes an increased synthesis of $1,25(OH)_2D_3$ in rat kidney in vitro (129). Thus, $1,25(OH)_2D_3$ is also a phosphate-regulating hormone that is functional during conditions of hypophosphatemia and when the parathyroid gland is suppressed (2).

In mammals, plasma concentrations of $1,25(OH)_2D_3$ are greatly increased during pregnancy and lactation (130,131). There is some suggestion that the sex hormones may play a role in directly regulating $25(OH)D_3$-1α-hydroxylase activity (132), but further work to elucidate the mechanisms by which these hormones may regulate the synthesis of $1,25(OH)_2D_3$ is necessary.

More controversial is the possible relationship between glucocorticoids and vitamin D. It has long been recognized that either long-term treatment with corticosteroids or long-term maintenance of supraphysiologic levels of these hormones can result in severe bone loss (133-135), and an alteration of vitamin D metabolism has been implicated as possibly being involved in this effect. This bone loss, characteristically more severe in bone with a higher content of trabecular bone (e.g. ribs and vertebrae) and less severe in bone with a higher content of less

metabolically active cortical bone (e.g., long bones), results from both decreased bone formation and increased bone resorption (136-139). Glucocorticoids apparently directly inhibit osteoblastic activity, both decreasing collagen synthesis by osteoblasts (140,141) and inhibiting conversion of precursor cells to functioning osteoblasts (141-143). On the other hand, corticosteroids inhibit osteoclastic activity in vitro (144,145); the increased bone resorption induced by corticosteroids seems to be an indirect effect of increased PTH secretion. Chronic corticosteroid administration in man is correlated with increased serum PTH (146,147), and parathyroidectomy abolishes the effect of corticosteroids on osteoclast activity and bone resorption (143).

PTH secretion is increased apparently as a result, at least partially, of a corticosteroid-induced decrease in intestinal calcium absorption (148-151). The basis for this decreased absorption, however, remains controversial (152). Initial work suggested that glucocorticoids might inhibit the conversion of vitamin D to $25(OH)D_3$ (153). Later studies demonstrated that cortisone impairs neither the formation of $25(OH)D_3$ nor its subsequent conversion to $1,25(OH)_2D_3$ (154,155). Serum $25(OH)D_3$ concentrations in patients with steroid-induced osteopenia are identical to those of matched controls (156), so if glucocorticoids do indeed interfere with vitamin D metabolism, the alteration must occur after the formation of $25(OH)D_3$ (152,154,156).

Some workers have suggested that corticosteroids can cause decreased formation or accumulation of $1,25(OH)_2D_3$ (157-159), but others have demonstrated that glucocorticoids can produce a significant inhibition of intestinal calcium absorption despite increased serum $1,25(OH)_2D_3$ concentrations (149). Still others have reported that

corticosteroids directly stimulate renal $25(OH)D_3$-1α-hydroxylase activity (160). Thus, it would appear that corticosteroids inhibit intestinal calcium absorption directly, perhaps by causing decreased synthesis of a carrier protein (152).

An alternative explanation is that corticosteroids interfere with $1,25(OH)_2D_3$ activity in target organs. Indeed, glucocorticoids were seen to decrease $1,25(OH)_2D_3$ receptor levels in cultured mouse bone cells (an effect dependent on the cellular growth cycle) (161) and in mouse intestine in vivo (162). There may, however, be a species and cell difference, since glucocorticoids prevented a decline of receptors in rat calvaria organ culture (163) and stimulated receptors in both rat osteoblast-like primary cultures (164) and rat intestine (165).

Because of all the conflicting data that have been reported, there is no general agreement as to whether either of these theories can completely explain the effect of corticosteroids on calcium metabolism or whether glucocorticoids can indeed alter renal 1-hydroxylase activity (166). It is clear, however, that steroid-induced osteopenia is a complex, multifactorial problem, and that the exact relationship between glucocorticoids and vitamin D and calcium metabolism remains obscure.

In addition to those mentioned above, six other factors have been suggested as possibly exerting some regulatory influence over the renal $25(OH)D_3$-1α-hydroxylase. These proposed factors include hydrogen ions (167-170), potassium (171,172), prolactin (173,174), growth hormone (175), calcitonin (176) and insulin (177-179). Clearly, much further work is necessary to elucidate completely the regulation of vitamin D metabolism.

BIOCHEMICAL BASIS OF VITAMIN D ACTIONS

As briefly described earlier, hormones in general are molecules produced at one site which can influence tissues at another site, reaching this distant site via blood transport. Hormones are of three types: (1) small molecules, derived from amino acids; (2) peptides or proteins; and (3) steroids, derived from cholesterol; they can elicit their specific cellular responses also in three ways: (1) by altering protein synthesis; (2) by altering enzyme catalytic activity; and (3) by altering membrane permeability (180). The peptide and amino acid-derived hormones, being water soluble, typically act by binding to specific receptors on cell surfaces and utilizing a second messenger intracellularly, often cAMP, to mediate the appropriate biologic response (181,182), usually an alteration of either enzymatic activity or a transport process (180)[2].

Steroid hormones, on the other hand, typically act by influencing gene expression. Since steroids are lipid soluble, they pass through the cell membrane and bind to specific cytosolic receptors (182). The hormone-receptor complex is then transferred to the nucleus where it influences transcription of a specific number of genes in an as yet unknown manner (182).

The vitamin D hormone

Much effort has been directed toward elucidating the biochemical mechanism of the action of $1,25(OH)_2D_3$. Experiments to locate the site

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[2]Unfortunately, as is often the case, this simplistic classification has major exceptions. The thyroid hormones (derivatives of the amino acid tyrosine), which enter the cell and stimulate RNA transcription and protein synthesis (183), are among the most notable examples.

of action at $1,25(OH)_2D_3$ within the cell have shown that at the time of response, $1,25(OH)_2D_3$ appears in the nuclear fraction in both intestinal epithelial cells (184) and osteoblasts (185) when these cells are differentially fractionated. Frozen section autoradiography using $26,27\text{-}[^3H]1,25(OH)_2D_3$ of high specific activity (186) has shown that labeled $1,25(OH)_2D_3$ is clearly localized in the nuclei of intestinal villi cells but not in goblet cells (187). This is seen prior to the calcium transport response, and a similar localization has been reported throughout the small intestine and in osteoblasts, parathyroid, gastric endocrine cells, certain cells of pituitary, skin epidermis and renal tubules (187,188).

Such a nuclear localization suggests that $1,25(OH)_2D_3$ acts as a classic steroid hormone, via a nuclear mechanism. Indeed, a cytosolic receptor for $1,25(OH)_2D_3$ has been identified in intestinal mucosa from rat (189), human (190) and chick (191), and in kidney (192-194), parathyroid (195), pituitary gland (196), bone (197-200), thymus (201), pancreas (194) and mammary tissue from rat (202) and human (203).

Further evidence for an effect of $1,25(OH)_2D_3$ on gene expression came with the observation that $1,25(OH)_2D_3$ specifically stimulates a Ca^{2+}-dependent accumulation of prolactin mRNA in the GH_4C_1 strain of rat pituitary cells (204). Previous <u>in vitro</u> experiments have demonstrated that $1,25(OH)_2D_3$ causes increased synthesis of prolactin by this same cell line (205). The authors suggest that $1,25(OH)_2D_3$ stimulates prolactin gene expression with a specific time course in a manner dependent on ionic calcium.

Vitamin D and intestine

More effort has been directed toward the study of the mechanism of vitamin D action in intestinal epithelium than in any other tissue. For example, the intestinal cytosolic vitamin D receptor has been well-studied and characterized. Scatchard analysis of the receptor (3.0-3.7 S) from chick intestine has shown a dissociation constant (Kd) of 5 x 10^{-11} M, consistent with the circulating concentration of $1,25(OH)_2D_3$ (30-100 pg/ml) (206). The Mr of the receptor by gel filtration is 70,000 (206).

The receptor-ligand complex is believed to be transferred to the nucleus since specific binding was seen to be localized to nuclear chromatin (207). The bound receptor binds to purified nuclei but the free receptor does not, and this binding is eliminated by partial digestion of intestinal nuclei with DNase I (207). The $1,25(OH)_2D_3$-receptor complex theoretically causes transcription of specific genetic information which codes for calcium and phosphate transfer proteins. In chick, an intestinal calcium binding protein (CaBP) was found, the production of which is dependent on vitamin D (208). De novo synthesis of both CaBP and CaBP mRNA is induced by administration of $1,25(OH)_2D_3$ (209-211), and synthesis of CaBP clearly occurs prior to the calcium uptake response to $1,25(OH)_2D_3$ in chick duodena in vitro (212). After uptake by the intestinal villus cell, calcium is then transferred by an as yet unknown mechanism and released at the basal lateral membrane in a sodium-dependent process (213).

An alternate mechanism for the effect of $1,25(OH)_2D_3$ on intestinal calcium transport has been proposed that is based, in part, on the observation that $1,25(OH)_2D_3$ also acts at the brush border membrane, an

observation that is clearly established (18). Goodman et al. (214) and Rasmussen et al. (215) suggest that the increase in calcium transport in response to $1,25(OH)_2D_3$ results from an alteration in membrane lipid composition. Indeed, the relative amounts of phosphatidylcholine and phosphatidylethanolamine (215), cholesterol esters and free cholesterol (214), as well as linoleic and arachidonic acids (216) all change in response to $1,25(OH)_2D_3$. In addition, it has been reported that essential fatty acid restriction inhibits the action of vitamin D on intestinal calcium transport (217), but others (218) have disputed this claim. Wasserman et al. (219) have suggested that $1,25(OH)_2D_3$ elicits a biphasic response in the intestine, the first at the brush border membrane and the second correlating with CaBP synthesis, and that both phases are required for the increased intestinal calcium absorption stimulated by $1,25(OH)_2D_3$. Exactly how these lipid changes might mediate calcium transport, however, remains to be elucidated.

Vitamin D, bone and bone proteins

Several lines of evidence from both in vivo and in vitro studies have suggested an interesting relationship between vitamin D and both bone metabolism and bone proteins. However, one must interpret in vivo data with some caution, since it is difficult to separate direct vitamin D effects from indirect effects due to alterations of calcium or phosphorous homeostasis.

Four metabolites of vitamin D_3, $25(OH)D_3$, $1,25(OH)_3D_3$, $24,25(OH)_2D_3$ and 1-hydroxyvitamin D_3, all stimulate bone resorption and inhibit collagen synthesis in fetal rat bone in organ culture, but $1,25(OH)_2D_3$ is approximately 1,000 times more potent than the others (220). These data suggest a single receptor species which binds $1,25(OH)_2D_3$ with high

affinity and other metabolites to a much lesser extent (220). In fetal rat calvaria in vitro, $1,25(OH)_2D_3$ in concentrations of 10^{-11} to 10^{-8} M decreases the biosynthesis of collagen by as much as 50%, an effect which is paralleled by a similar action on intracellular procollagen mRNA levels (221). This response is almost identical to a similar effect of PTH on collagen and procollagen mRNA synthesis in fetal rat calvaria in vitro (222). These data also seem to suggest that $1,25-(OH)_2D_3$ functions at the level of mRNA transcription.

The bone resorption stimulated by vitamin D may be mediated by an effect of $1,25(OH)_2D_3$ on cell differentiation or osteoclast development. It has been reported that $1,25(OH)_2D_3$ induces myeloid leukemia cells to differentiate into macrophages (223-225), the presumed progenitor cells of osteoclasts (226-229). Indeed, monocytes and macrophages may directly cause bone resorption independently of osteoclastic activity (230). Miyaura et al. (231) have reported that $1,25(OH)_2D_3$ suppresses the proliferation of a normal bone marrow granulocyte-macrophage progenitor cell line, suggesting that there may be specific receptors for $1,25-(OH)_2D_3$ in hematopoietic stem cells or immature myeloid cells. The question of whether $1,25(OH)_2D_3$ is involved in the differentiation of normal hematopoietic stem cells is currently being studied in this same laboratory (231). Other workers have reported the direct regulation of macrophage activity (232) and the induction of monocytic differentiation (233) by $1,25(OH)_2D_3$. Certainly, the intriguing notion of a role for $1,25(OH)_2D_3$ in the control of cellular differentiation and development warrants further investigation.

Bone collagen may be structurally regulated by vitamin D. In vitamin D-deficient, [^{3}H]$NaBH_4$-reduced chick bone collagen, hydroxylation of lysine residues is increased, yielding an increased ratio of the reduced crosslinks dihydroxylysinenorleucine (DHLNL) to hydroxylysinenorleucine (HLNL) (234). Profiles of reducible crosslinks of vitamin D-deficient bone are characteristic of immature collagen (high DHLNL/HLNL ratio) (235,236). However, collagen from vitamin D-deficient bone shows an increased quantity of the stable crosslink pyridinoline, considered characteristic of mature collagen (237). It has been proposed that DHLNL may participate as an intermediate in pyridinoline formation (238,239) and that the lack of complete mineralization of collagen in vitamin D-deficient bone may permit the conversion of reducible, nonstable crosslinks to nonreducible, pyridinoline crosslinks (237).

In a second study with vitamin D-deficient chicks, the increased ratio of the reduced crosslinks DHLNL/HLNL in bone collagen as outlined above occurred prior to changes in growth rate and could not be correlated with lysine hydroxylation or hypocalcemia (240). The author suggests that this type of crosslink analysis provides one of the earliest indications of a bone disturbance due to vitamin D deficiency and that vitamin D specifically acts to increase the rate of the maturation of bone collagen.

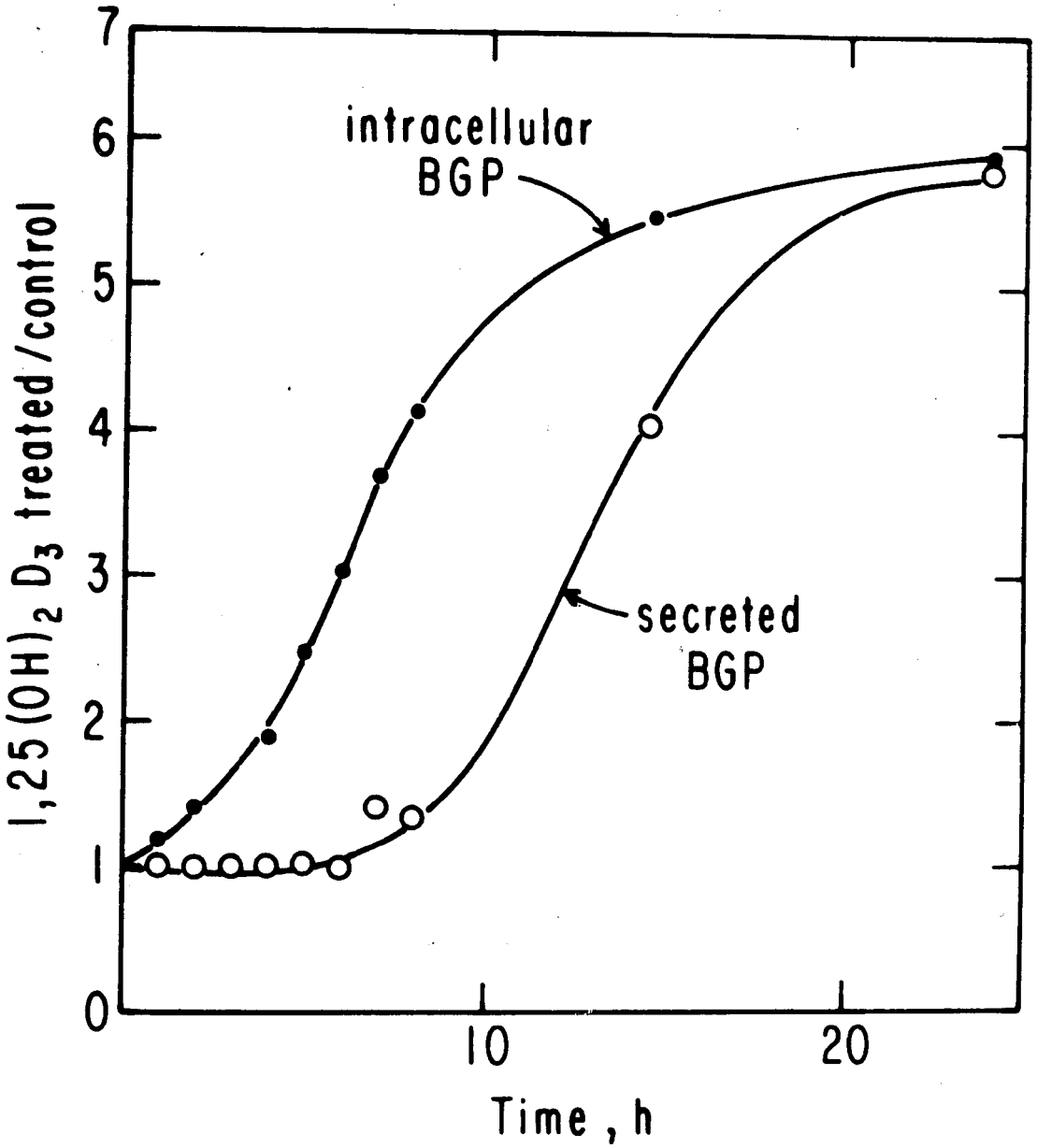
Noncollagenous proteins of bone also show alterations due to vitamin D. Bone matrix of vitamin D-deficient, rachitic chicks shows a significantly decreased content of phosphoprotein as determined by a decrease in both the total amount and the concentration of protein-bound o-phosphoserine and o-phosphothreonine (241).

It has been reported that proteoglycans isolated from growth cartilage of rachitic chicks are of smaller size than those isolated from controls, but this could be related to calcium deficiency rather than vitamin D status (242). The physiological importance of this size change is unknown.

Another group of proteins which has been suggested as having a role in either the formation or regulation of mineralized tissues are the γ-carboxyglutamic acid (Gla)-containing proteins contained in these mineralized tissues. A major Gla-containing protein present in the organic matrix of bone, known as bone Gla protein (BGP) (243,244) or osteocalcin (245), is the first known bone protein the biosynthesis of which is specifically increased in response to $1,25(OH)_2D_3$. Intravenous injection of $1,25(OH)_2D_3$ increases the serum concentration of BGP in rats (246), an effect which parallels almost identically a similar effect seen in earlier studies on intestinal CaBP. $1,25(OH)_2D_3$ causes a 6-fold increase in the synthesis of BGP by rat osteosarcoma cells cultured in vitro (Fig. 5), and this response is dose-dependent (247).

Vitamin D may be related to BGP in another manner. Hypomineralized cortical bone from rachitic chickens contains significantly increased quantities of Gla-containing proteins when compared to bone from control chickens (248), and this increase in Gla content is inversely dependent on the degree to which the bone is mineralized (249). In contrast, however, the BGP concentration is decreased by 50% in rachitic bone (249). A distinction must be made between assays that measure total Gla content and those that measure BGP. Total Gla by amino acid analysis identifies the total number of Gla residues, regardless of the protein of origin. Assays for immunoreactive BGP, on the other hand, identify a

Fig. 5. Stimulation of BGP synthesis and secretion by $1,25(OH)_2D_3$. The media of confluent 60-mm culture plates were exchanged for the same media or media containing 1 ng/ml of $1,25(OH)_2D_3$ at time zero. Two experimental and two control plates were terminated at each time point and analyzed for intracellular and media levels of BGP. Each point is the average experimental value divided by the average control. (Reprinted from Price PA, Baukol SA. J Biol Chem 1980: 255: 11660, with permission from authors.)


intracellular
BGP
secreted
BGP
1,25(OH)2 D3 treated/control
Time, h
0
1
2
3
4
5
6
7
10
20

specific, low molecular weight protein which contains three Gla residues (245). The above results have prompted the authors to suggest that vitamin D may regulate the biosynthesis of BGP either directly, as above, or indirectly by activating an enzymatic processing of a putative, higher molecular weight BGP precursor to yield a functional BGP (249).

Another molecule, which was very early suggested as possibly being involved in the mineralization process, has been related to $1,25(OH)_2D_3$ in a manner that is strikingly similar to that described for BGP. Citrate, present in bone in abundant quantities (250), can interact strongly with both calcium (251) and hydroxyapatite (252) as a result of its highly anionic nature. Many studies in the 1940's and 1950's have shown that citrate levels in bone are increased by both vitamin D and PTH (253), but the subject is for the most part curiously absent in the later literature. The notion of a possible mineral regulatory function for this organic acid has recently been revived by the demonstration that $1,25(OH)_2D_3$ increases citrate secretion by rat osteosarcoma cells (253), the same cell line in which had been demonstrated the $1,25(OH)_2D_3$-induced stimulation of BGP synthesis described above. On the basis of these findings, the authors suggest that citrate, perhaps along with BGP, may play a role in mediating the calcium mobilization effect of $1,25(OH)_2D_3$, perhaps by inhibiting bone mineralization (253).

Finally, it has been suggested that vitamin D may regulate alkaline phosphatase activity in bone (254). Although the function of alkaline phosphatase in bone is unknown, the skeletal isoenzyme has long been thought to play a role in bone formation (255,256); presumably, alkaline phosphatase is a marker for osteoblastic activity (257). $1,25(OH)_2D_3$

has been reported to cause an increase in alkaline phosphatase activity in two human osteosarcoma cell lines, but neither $25(OH)D_3$ nor $24,25-(OH)_2D_3$ showed this same effect (258).

VITAMIN D AND BONE PATHOLOGY

Several diseases of bone can theoretically be traced to aberrations in the function of the vitamin D system. In addition, inborn genetic disorders of vitamin D metabolism can be manifested as pathological changes in the skeletal system.

Renal osteodystrophy

Renal osteodystrophy, routinely seen as a complication of chronic renal disease, is a disease which presents with severe osseous abnormalities. Its effects are most evident in children. The term renal osteodystrophy arose to refer collectively to the bony changes seen in uremia (259), and the biochemical basis for these changes is now becoming more apparent. The clinical course of the disease can theoretically be traced to alterations in the vitamin D endocrine system. Functional loss of renal nephrons leads to both a deficiency of $1,25(OH)_2D_3$ and a retention of circulating phosphate. Lack of $1,25(OH)_2D_3$ results in a failure to absorb calcium properly. The resultant hypocalcemia stimulates the parathyroid gland, leading to a secondary hyperparathyroidism and lesions of excessive bone resorption. A severe rickets or osteomalacia can result, as well as osteitis fibrosa or osteosclerosis, both of which can result from high circulating PTH. The decrease in both calcium absorption and mineralization of bone further intensifies the secondary hyperparathyroidism (260). Controversy exists, however, as to the relative contributions of phosphate retention or decreased

$1,25(OH)_2D_3$ production to the clinical picture of renal osteodystrophy (261,262).

Clinical management of renal osteodystrophy includes the use of phosphate binders (aluminum hydroxide, calcium carbonate) to control phosphate intake, helping to maintain normal serum phosphate concentrations, and then treatment with $1,25(OH)_2D_3$ (0.25-2.0 µg/d) (263). Some authors have suggested that a combined treatment with both $25(OH)D_3$ (0.5-0.75 µg/d) and $1,25(OH)_2D_3$ (0.5-2.0 µg/d) is more efficacious than treatment with $1,25(OH)_2D_3$ alone (264). One must use caution, however, since aluminum itself has also been implicated in the pathogenesis of uremic bone disease (265).

Osteoporosis

Osteoporosis, another abnormality of bone and seen routinely with aging and often much more rapidly in post-menopausal females, is a complex, multi-factorial disease. One cause is decreased calcium absorption. Some investigators (266) have correlated this malabsorption with decreased circulating levels of $1,25(OH)_2D_3$, but others (267,268) have disputed this claim. Nonetheless, calcium absorption is markedly improved with administration of small doses of $1,25(OH)_2D_3$. Treatment of post-menopausal, osteoporotic females with $1,25(OH)_2D_3$ (0.5 µg/day) as a single dose greatly improves calcium balance (269). However, there are no data to suggest that bone mass increases with this treatment (261). In the treatment of post-menopausal osteoporosis in females, it is currently suggested that a combined therapy of $1,25(OH)_2D_3$ and either estrogen (15-25 µg/d) or norethisterone (2.5-5.0 mg/d) is the treatment of choice (268). Clinical trials are currently in progress to test the usefulness of $1,25(OH)_2D_3$ in the treatment of osteoporosis (261).

Vitamin D-dependent rickets type I

Vitamin D-dependent rickets type I is a heritable, autosomal recessive derangement of vitamin D metabolism in which the renal 25(OH)-D_3-1α-hydroxylase enzyme is either deficient or defective (270). Clinical signs and symptoms include all those of classical rickets, and patients show reduced blood concentrations of $1,25(OH)_2D_3$, calcium and phosphorous (271). Treatment with small doses of $1,25(OH)_2D_3$ (0.05 μg/kg twice/d) yields dramatic improvement of the disease (272). Massive doses of $25(OH)D_3$ will apparently yield similar results (270,271).

Vitamin D-dependent rickets type II

Vitamin D-dependent rickets type II is also a heritable, autosomal recessive disorder of vitamin D metabolism. The defect is resistance of target organs to $1,25(OH)_2D_3$ (273,274). The clinical picture is one of severe rickets, an elevated blood concentration of $1,25(OH)_2D_3$ and hair loss. Treatment is difficult but includes high doses of calcium, phosphorous and $1,25(OH)_2D_3$ (271).

CONCLUDING REMARKS

In conclusion, great progress has been made in the understanding of vitamin D. Vitamin D has been elevated from being the poorly understood antirachitic factor present in fish liver oils to playing a central role in the intricate regulation of calcium and phosphorous homeostasis. Indeed, the prospects for future clinical use of vitamin D metabolites in medicine seem bright. However, much research is still required in order to define completely the vitamin D endocrine system.

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APPEARANCE OF DENTIN γ-CARBOXYGLUTAMIC ACID-CONTAINING PROTEINS IN DEVELOPING RAT MOLARS *IN VITRO*

by

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ABSTRACT

An *in vitro* model of mineralization was devised in order to study the developmental appearance of dentin γ-carboxyglutamic acid-containing proteins (DGPs) in relation to the onset of mineralization. Maxillary third molars from 11-day-old rats were cultured with or without fetal calf serum (FCS) as modified from Navia *et al*. (1984). Molars were incubated without radiolabel, or with either $^{45}CaCl_2$ (5 μCi/ml) for 24 hr at various stages of a ten-day culture period or [^{3}H]-leucine (10 μCi/ml) for 24 hr at the eighth day of culture. Molars were lyophilized and extracted with 10% formic acid overnight at 4°C. DGPs in extracts were detected by immunologic and chromatographic techniques; DGPs in molar sections were detected by immunolocalization using indirect immunofluorescence. Molar development was evaluated histologically using the Von Kossa staining technique.

Molars cultured with FCS showed histologic evidence for mineralized dentin and enamel and a significant increase in ^{45}Ca uptake after the sixth day *in vitro*. Eleven-day-old molars *in vivo* and molars cultured without FCS showed no evidence of the presence of mineralized tissues. [^{3}H]-Leucine-labeled DGPs were isolated and identified by affinity and reversed-phase high-performance liquid chromatography and by gel electrophoresis from both mineralized and unmineralized molars. DGP antigens were localized immunohistochemically using rabbit anti-rat antibodies raised against a highly purified DGP preparation. In the unmineralized molar, antigenicity was seen in odontoblasts but not in predentin matrix, preodontoblasts, or in any other cell type. Antigens in the mineralized molar were localized to odontoblasts and dentin. Analysis of these data indicates that rat molars cultured with FCS show *de novo*

mineralization *in vitro* and suggests that rat molar odontoblasts synthesize DGPs concurrently with the elaboration of predentin matrix but independently of mineral deposition.

INTRODUCTION

γ-Carboxyglutamic acid (Gla) is an unique amino acid formed by a vitamin K-dependent post-translational modification of specific glutamate residues (Esmon et al., 1975). Since first being discovered in prothrombin and blood coagulation factors VII, IX, and X (Stenflo et al., 1974), Gla has been identified in other proteins from several sites, including serum (Price and Nishimoto, 1980), bone matrix (Hauschka et al., 1975; Price et al., 1976), dentin (Linde et al., 1980), and other mineralized or calcified tissues (Lian et al., 1976, 1977; Levy et al., 1979; Hamilton et al., 1982). An abundant Ca^{2+}-binding, Gla-containing protein of bone (molecular weight = 5,200 - 5,900) has been termed "osteocalcin" (Hauschka and Gallop, 1977) or "bone Gla protein" (BGP, Price et al., 1980) and contains three or, less frequently, two Gla residues per molecule. BGP has been the subject of extensive investigations, but its biological function remains obscure. The Gla-containing proteins of dentin (DGPs, Bronckers et al., 1985) are highly homologous to those from bone and also contain three Gla residues per molecule (Linde et al., 1980, 1982). However, DGPs appear to be heterogeneous and may represent a family of closely related macromolecules (Linde et al., 1982).

This study was designed to address a developmental question - when are DGPs first synthesized relative to the onset of mineralization? This question has been studied regarding bone, but the answer remains equivocal. Gla residues first appear approximately at the time of first mineralization in chick embryonic long bone (Hauschka and Reid, 1978) and in ectopic bone induced in the rat by implantation of demineralized bone powder (Hauschka and Reddi, 1980). BGP could not be detected in

fetal rat bone by radio-immunoassay (RIA, Price et al., 1980), but Hauschka (1979) used high-performance liquid chromatography (HPLC) to identify osteocalcin in bone from six-month-old fetal calf and from 17-day-old chick embryo, with the osteocalcin apparently appearing concurrently with the first detectable mineral. Dentinogenesis - a well-ordered progression of histodifferentiation, morphodifferentiation, extracellular matrix (predentin) elaboration, and, finally, mineralization to form mature dentin - appears to be an ideal model for such a study. However, this question of the developmental appearance of Gla-containing proteins has not previously been addressed with regard to dentin.

The rat molar organ was utilized to develop an in vitro model of mineralization that would be useful for studies regarding the physiological significance of DGPs. This model was then used for initial investigations concerning the developmental appearance of DGPs.

MATERIALS AND METHODS

Molar organ culture. - Molar organs were cultured essentially according to Navia et al. (1984) with modifications. Briefly, maxillary third molars were removed from 11-day-old rat pups[1] and placed on filters[2] supported at the liquid-gas interface in multi-well plastic dishes[3]. Molars were cultured in minimum essential medium[4] as modified from Yamada et al. (1980), supplemented with L-glycine[5] (0.67 mM), L-glutamine[6] (2.05 mM), penicillin-G, sodium salt[5] (0.34 mM), streptomycin sulfate[5] (0.069 mM), Hepes buffer[7] (25.18 mM), and ascorbic acid[8] (0.142 mM, added fresh daily) with or without supplementation with 10% fetal calf serum[6] (±FCS). Molars were cultured for periods of up to ten days at 37°C, with daily media changes in a humidified atmosphere of 50% O_2, 45% N_2, and 5% CO_2 (Wigglesworth, 1968; Bronckers et al., 1981, 1983).

Radiolabeling. - Molars were incubated in the presence of $^{45}CaCl_2$[9] (5 µCi/ml, 1.96 mCi/µg) or [^{3}H]-leucine[10] (10 µCi/ml, > 110 Ci/mmol) for 24 hr. At the end of the labeling period, molars (in the case of ^{45}Ca) were washed three times in sterile, unlabeled medium to remove adsorbed radiolabel or (in the case of ^3H) were incubated for one hr in fresh, unlabeled medium.

Biochemical analysis. - After the culture period, molars were harvested and immediately lyophilized. Molars radiolabeled with ^{45}Ca

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[1] Sprague-Dawley-derived, Charles River Laboratories, Wilmington, MA
[2] 0.45-µm pore size, HABG 01300, Millipore Corporation, Bedford, MA
[3] Corning Cell Wells 25820, Corning Glass Works, Corning, NY
[4] Eagle, 410-1100, Gibco Laboratories, Grand Island, NY
[5] Sigma Chemical Co., St. Louis, MO
[6] Gibco Laboratories, Grand Island, NY
[7] N-2-Hydroxyethyl piperazine N'-2-ethane sulfonic acid[5]
[8] MCB Manufacturing, Cincinnati, OH
[9] Amersham International, Amersham, UK
[10] ICN Radiochemicals, Irvine, CA

were weighed[11] individually and extracted with 0.5 ml of 10% formic acid for 24 hr, with shaking, at 4°C. This extract was collected, and molars were washed with a second 0.5 ml of cold 10% formic acid. This 0.5 ml was added to the first, and radioactivity in 1-ml samples was counted[12] using a liquid scintillation cocktail[13].

Molars radiolabeled with [^{3}H]-leucine were pooled, lyophilized, and extracted collectively with 10% formic acid as above. Extracts were chromatographed on a column (10 cm x 0.9 cm) of Sephadex G-25[14] (fine), eluted with 10 mM sodium phosphate buffer, pH 7.4, containing protease inhibitors (Linde et al., 1980) to remove unincorporated label. Radiolabeled DGPs were isolated by affinity chromatography and identified by HPLC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

Antibody production and affinity chromatography. - Antiserum was prepared essentially as described by DiMuzio et al. (1983). A highly purified DGP preparation, DGP-γ_3 (Linde et al., 1982), was used for all antigen injections. Briefly, antigen (0.5 mg) dissolved in 0.5 ml of 0.01 M Tris[5]/HCl, pH 7.4, and 0.5 ml of Freund's adjuvant[6] was injected subcutaneously into two sites on the back of a female rabbit[15]. The rabbit was bled prior to the first injection to obtain pre-immune serum. Injections were at 14-day intervals, with the rabbit bled seven days after each injection. The appearance of antibody was detected by enzyme-linked immunosorbent assay (ELISA, Baker et al., 1982). Rabbit

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[11]Cahn Electrobalance, Model G, Cahn Instrument Co., Cerritos, CA
[12]LS 8000, Beckman Instruments, Inc., Fullerton, CA
[13]Scintiverse I, Fisher Scientific Co., Fair Lawn, NJ
[14]Pharmacia, Uppsala, Sweden
[15]New Zealand White, Myrtle Rabbitry, Thompson Station, TN

anti-rat DGP-γ_3 antibody (anti-DGP) activity in serum was first detected by ELISA after the third injection of antigen.

Affinity columns were prepared as described by DiMuzio et al. (1983) by linking purified protein to a derived, activated agarose[16]. Anti-DGP, purified by chromatography on an affinity column prepared with rat DGP-γ_3, was used as the protein ligand. A small affinity column (approximately 0.5 ml total volume) was prepared with anti-DGP and equilibrated in 0.15 M NaCl/0.01 M sodium phosphate buffer, pH 7.4, containing 0.03% NaN_3 (equilibration buffer). Non-specific binding sites were blocked by passage of 5% bovine serum albumin[5] in equilibration buffer.

Samples (0.5 ml) were applied to the anti-DGP affinity column and incubated for one hr at 24°C. Unbound proteins were eluted with equilibration buffer; bound proteins were eluted with 4 M of guanidine hydrochloride in the same buffer. Radio-iodinated samples were introduced to the affinity column after passage through a pre-column prepared with pre-immune rabbit immunoglobulins and elution with equilibration buffer. Proteins were de-salted and lyophilized for subsequent analysis.

Radio-iodination. - [^{125}I]-Iodination of proteins was performed as described by DiMuzio et al. (1983), using chloramine-T[5] (N-chloro-p-toluenesulfonamide, sodium salt) as the oxidant. Protein (50 µg) was iodinated with 1.0 mCi of $Na^{125}I$[9] (13-17 mCi/µg). Free $Na^{125}I$ was removed by chromatography on Sephadex G-25 (fine, 10 cm x 0.9 cm), and fractions containing incorporated radiolabel were pooled and stored at -20°C.

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[16]Affi-Gel-10, Bio-Rad Laboratories, Richmond, CA

Reversed-phase chromatography. - Samples were dissolved in 150 µl of eluant (1% trifluoroacetate, TFA) separated by HPLC on a reversed-phase column[17] (3.9 mm x 30 cm) eluted with 1% TFA (1.5 ml/min) and a gradient of 0 - 45% acetonitrile (ACN) for 30 min, and subsequently held constant at 45% ACN. Fractions were collected every 30 sec, and radioactivity was detected as described above (for ^{3}H) or on an automatic gamma counter[18] (for ^{125}I). Peak [^{3}H]-leucine-labeled fractions were pooled, lyophilized, electrophoresed on polyacrylamide slab gels, and detected by fluorographic techniques (described below).

Electrophoresis, autoradiography, and fluorography. - Proteins were separated by SDS-PAGE using 1.5 mm, 5%-15% gradient gels essentially as described by Laemmli (1970) and modified by Butler et al. (1981).

After electrophoresis, gels with [^{125}I]-labeled proteins were vacuum-dried and exposed with X-ray film[19] at -20°C using an intensifying screen[20] for from 30 to 90 min. Gels with [^{3}H]-labeled proteins were processed for fluorography using 22% PPO[5] (2,5-diphenyloxazole) in dimethyl sulfoxide[21] as the scintillant, vacuum-dried, and exposed as above for from four to six wk.

Histology. - Molars were overlaid with 1% bacto-agar[22], fixed in 10% neutral buffered formalin for 48 hr, paraffin[23]-embedded, and sectioned (6 µm thick). Sections were stained for mineral by the Von Kossa technique (Sheehan and Hrapchak, 1980), counter-stained with nuclear fast red[5], and examined by light microscopy.

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[17] C-18 µBondapak, Waters Associates, Inc., Milford, MA
[18] LKB-Wallac Clini Gamma, Wallac Oy, Turku, Finland
[19] KP 71650C, Kodak Blue Brand Film, Eastman Kodak Co., Rochester, NY
[20] Cronex Xtra Life, DuPont Co., Newtown, CT
[21] Pierce Chemical Co., Rockford, IL
[22] Difco Laboratories, Detroit, MI
[23] Tissue Prep, Fisher Scientific Co., Fair Lawn, NJ

Immunohistochemistry. - Cultured molars were evaluated as described by Bronckers et al. (1985) with modifications. Briefly, molars removed from culture were fixed in freshly prepared 5% paraformaldehyde[24] in 0.1 M sodium phosphate buffer, pH 7.3, at 4°C for 3.5 hr. Molars were rinsed in cold phosphate-buffered saline[25] (PBS), dehydrated, xylene-washed, paraffin-embedded, and sectioned (6 μm thick).

Paraffin was removed from sections with xylene, and sections were rehydrated and then rinsed in cold PBS. Sections were incubated in a humidified chamber with a 1:50 dilution of anti-DGP antiserum (primary antibody) or pre-immune serum (control) in PBS at 24°C for one hr or at 4°C overnight. After incubation, sections were rinsed in cold PBS and incubated with secondary antibody, fluorescein isothiocyanate (FITC)-linked goat anti-rabbit immunoglobulins[26] (1:10 working dilution, one hr, 24°C). Sections were rinsed in PBS, mounted in glycerol, and examined by ultraviolet light microscopy[27].

Statistical evaluation. - Significance of differences in ^{45}Ca-uptake or dry-weight between groups at each day was determined by Student's *t* test; significance of the increase in ^{45}Ca-uptake or dry weight for each group was determined by a one-way analysis of variance (Snedecor and Cochran, 1967), with specific sites of differences among means determined by multiple means comparison (Duncan, 1955). Significance of molar growth as indicated by dry weight was determined by linear regression analysis (Snedecor and Cochran, 1967).

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[24] Polysciences, Inc., Warrington, PA
[25] Dulbecco's, 420[6]
[26] 645901, Calbiochem-Behring, San Diego, CA
[27] Dialux 20, E. Leitz, Inc., Rockleigh, NJ

RESULTS

Histological evaluation of mineralization. - Maxillary third molars excised from 11-day-old rat pups showed an enamel organ enclosing the dental papilla (Fig. 1a). Preodontoblasts and cells of the inner enamel epithelium were present (Fig. 1b). Predentin matrix was occasionally seen at a cusp tip area with associated odontoblasts (not shown), but in no sections studied were there any areas of mineralized dentin or enamel as revealed by Von Kossa staining.

Molars cultured for ten days in the presence of 10% FCS showed continued cuspal development and well-formed odontoblast and ameloblast cell layers (Figs. 1c and 1d). Predentin matrix was present, along with sites of dentin and enamel showing heavy Von Kossa staining.

Molars cultured for ten days in defined medium without serum supplementation showed continued cuspal formation with pronounced odontoblast and ameloblast cell layers (Figs. 1e and 1f). Although predentin matrix was abundant, there was no evidence of any site that stained by the Von Kossa reaction (i.e., no areas of mineralized tissues).

Biochemical evaluation of mineralization. - Eleven-day-old rat maxillary third molars, cultured in defined medium without serum supplementation, showed a slight increase in ^{45}Ca uptake over the ten-day culture period, while molars cultured in medium supplemented with 10% FCS showed a significant ($p<0.01$) and steady increase in uptake of radiolabel after the sixth day *in vitro* (Fig. 2A). Incorporation of radiolabel by molars cultured with FCS was significantly higher than that by molars cultured without FCS by the sixth day *in vitro*.

Fig. 1 - Sections of rat maxillary third molars stained with Von Kossa/nuclear fast red. (a) Eleven-day-old molar in vivo, eo, enamel organ; dp, dental papilla. Bar = 30 µm x 234.8. (b) Higher power view of (a). IEE, inner enamel epithelium; po, pre-odontoblasts. Bar = 10 µm x 587.0. (c) Eleven-day-old molar, cultured for ten days with FCS. a, ameloblasts; m, mineralized tissue; o, odontoblasts; p, pulp. Bar = 30 µm x 238.4. (d) Higher power view of (c). d, dentin; e, enamel; pd, predentin. Bar = 10 µm x 587.0. (e) Eleven-day-old molar cultured for ten days without FCS. Bar = 30 µm x 234.8. (f) Higher power view of (e). Bar = 10 µm x 587.0.

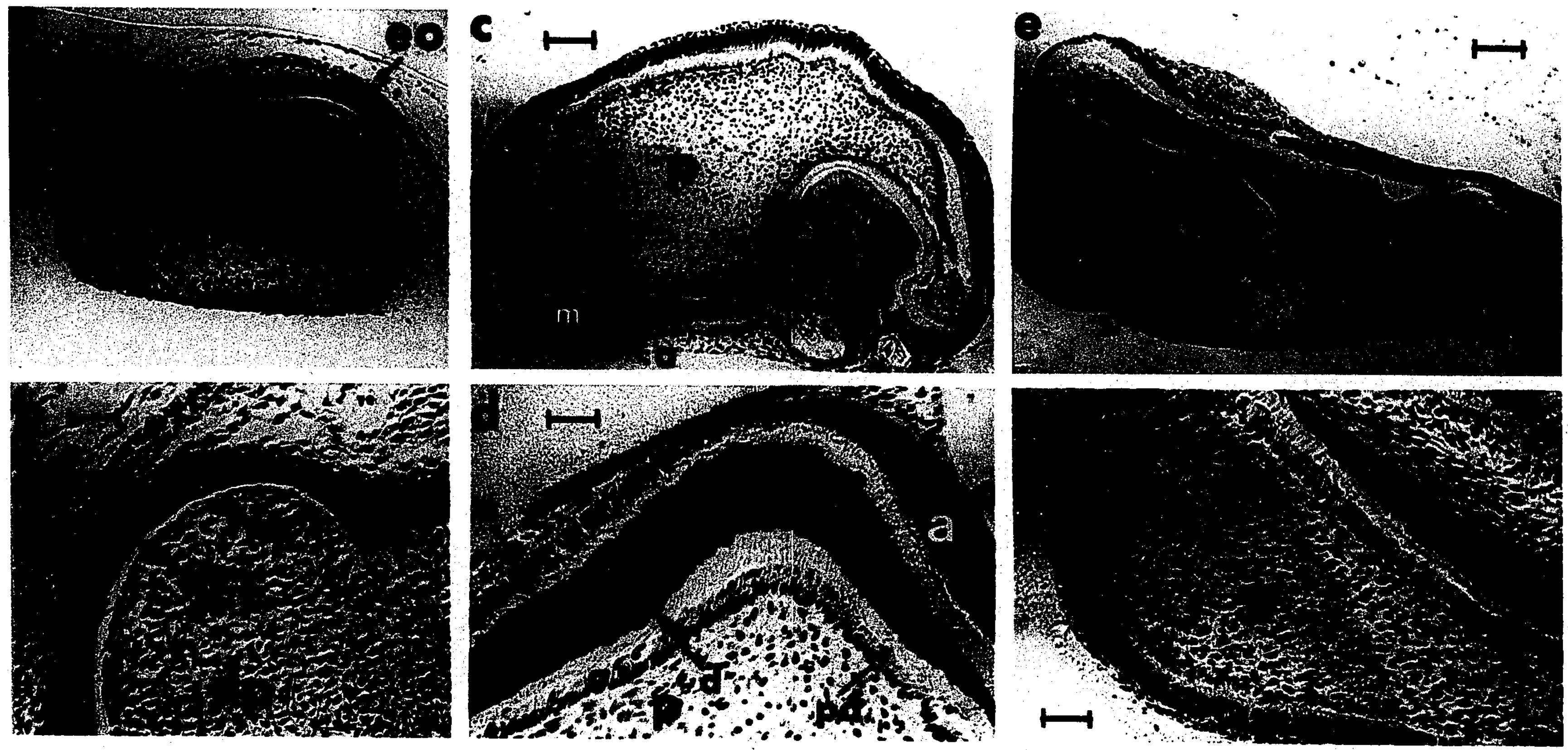
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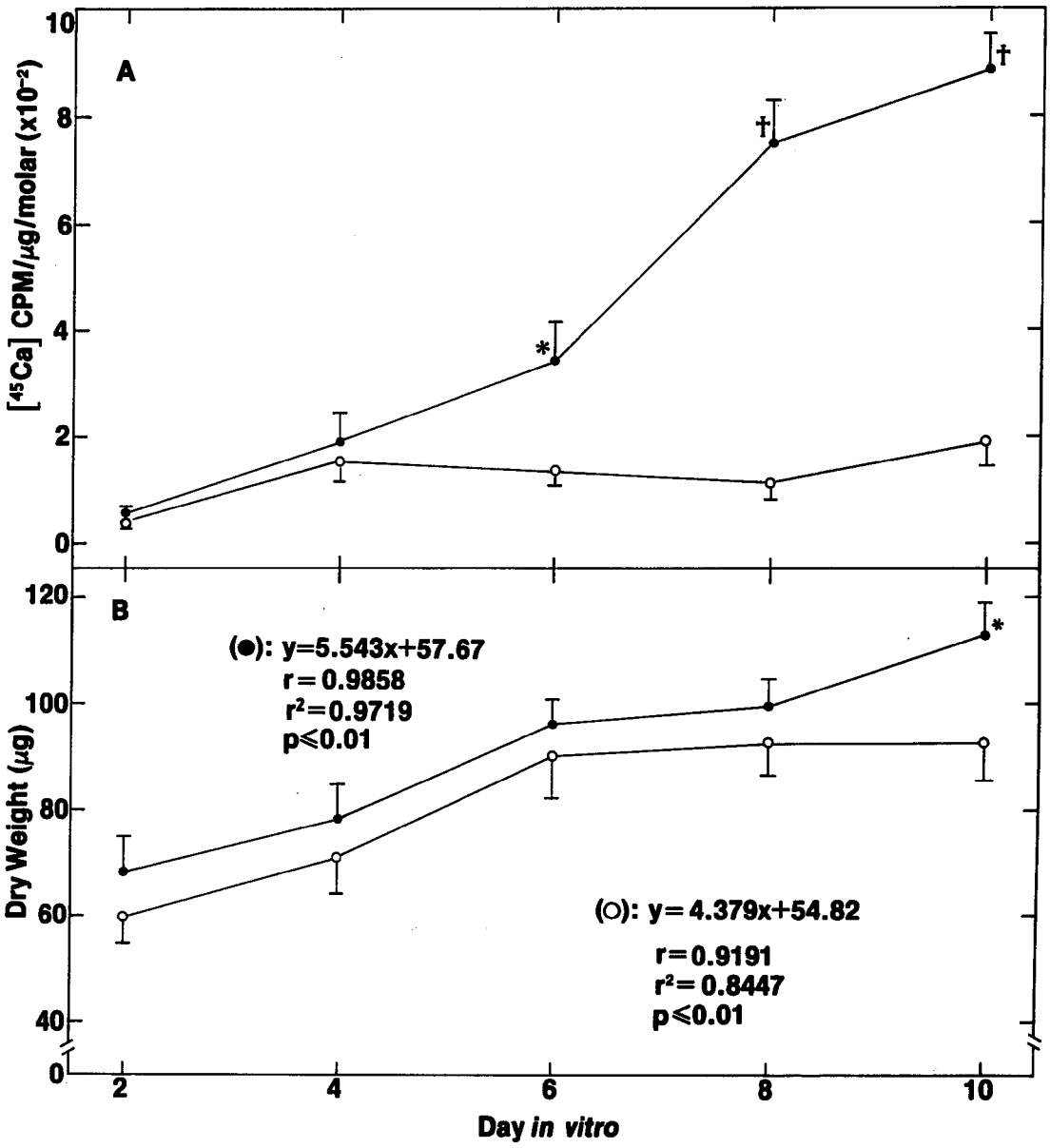
Fig. 2 - Parameters of *in vitro* molar organ development. Molars were incubated (± FCS) with ^{45}Ca (5 μCi/ml) for 24 hr at the times specified. Molars were lyophilized, weighed, and extracted for 24 hr with 10% formic acid. Each point represents the average of from 22 to 24 molars ± SEM. (A) ^{45}Ca uptake per dry weight per molar per day *in vitro*. (B) Dry weight per molar per day *in vitro*. (•) molars cultured with FCS; (o) molars cultured without FCS. * = significantly different from -FCS group, $p < 0.05$. † = significantly different from -FCS group, $p < 0.001$.

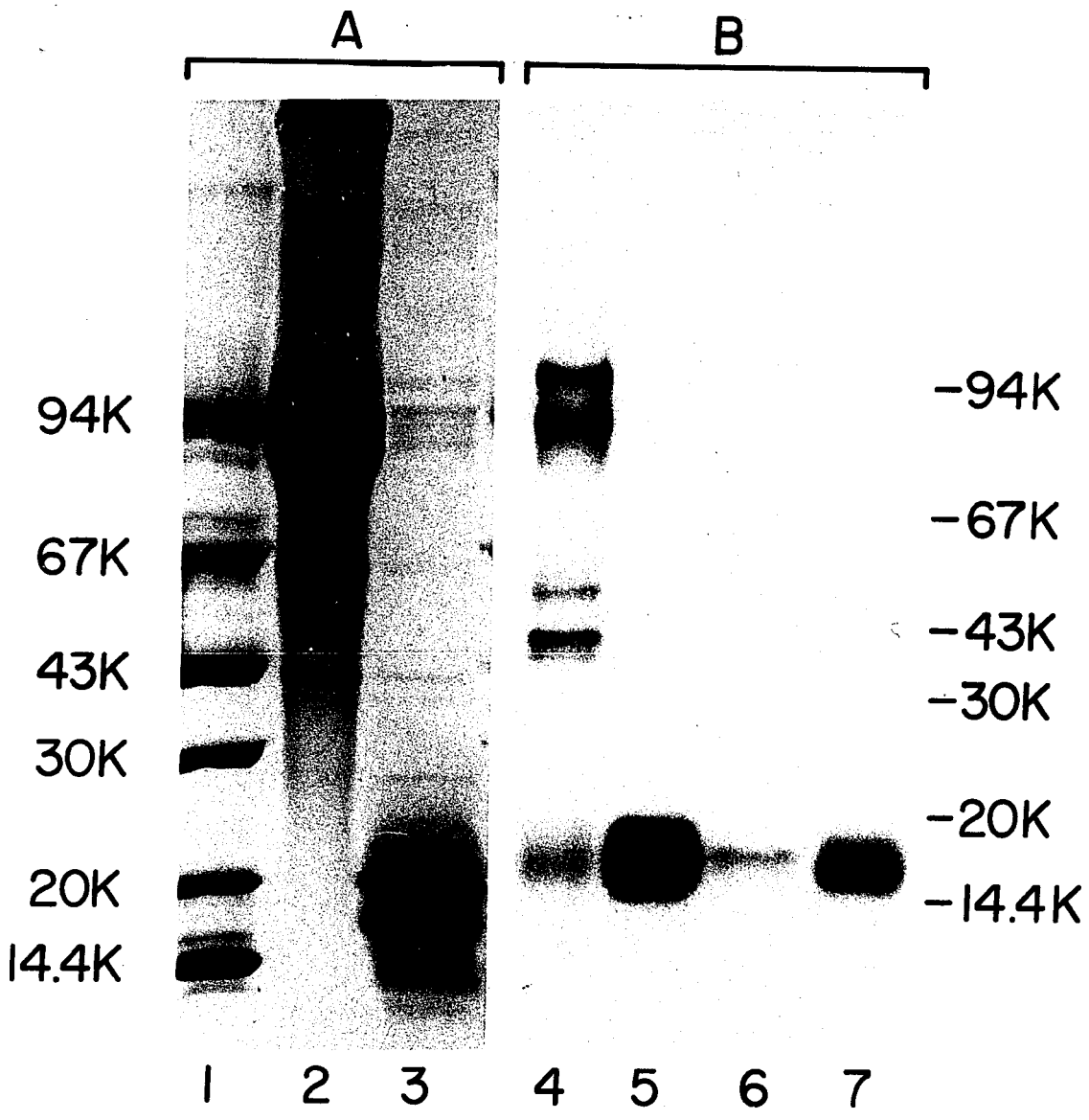

A
B
[45Ca] CPM/μg/molar (x10-2)
Dry Weight (μg)
Day in vitro
(●): y=5.543x+57.67
r=0.9858
r2=0.9719
p≤0.01
(○): y=4.379x+54.82
r=0.9191
r2=0.8447
p≤0.01

Molars from both groups (± FCS) showed a significant trend of increasing dry weight over the culture period (Fig. 2B); there was a significant increase in weight between days 4 and 6 *in vitro* ($p<0.01$, with overlap in the -FCS group; data not shown). Molars cultured with FCS showed a greater rate of weight gain than did molars cultured in serumless medium. In addition, there was a significant difference in dry weight between the two groups at day 10 *in vitro*.

Characterization of antibody and affinity chromatography. - A competitive binding RIA has indicated that anti-DGP antibody does not cross-react with any other rat dentin non-collagenous proteins (NCPs) and binds DGP-γ_3 with a half-maximal value at a concentration of 1 ng/400 µl (unpublished data). In order to characterize the anti-DGP affinity column, an [^{125}I]-labeled total extract of rat incisor dentin NCPs, prepared according to Linde *et al*. (1980), was passed through the column. Unbound and bound fractions were resolved by SDS-PAGE and detected by autoradiography. Fig. 3 (lanes 4 and 5) showed that the anti-DGP column selectively bound DGPs from a total extract of dentin NCPs (lanes 2 and 3), which co-migrated with heterogeneous DGPs (lane 7) and in the region of DGP-γ_3 (lane 6).

In vitro biosynthesis of DGPs. - Radiolabeled proteins extracted from molars incubated *in vitro* with [^{3}H]-leucine were fractionated by anti-DGP affinity chromatography. Bound fractions with high radioactivity were pooled and separated by reversed-phase HPLC. Figs. 4a and 4b show that labeled proteins from molars of both groups (±FCS) co-eluted with purified [^{125}I]-DGP-γ_3 standard. Fig. 4 (inset) shows that molar material from the DGP peak in Fig. 4a (lane 1) and material from a DGP peak similar to that of Fig. 4b (lane 2) migrated in the position of

Fig. 3 - (A) SDS-polyacrylamide gel electrophoresis of Sephacryl-200 fractions of total non-collagenous protein (NCP) extracts from adult rat dentin. Proteins stained with Coomassie Brilliant blue[28]. (Lane 1). Molecular weight standards[14] phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and α-lactalbumin (14,400). (Lane 2) Total NCP extract Sephacryl 200 void volume (DGP-depleted fraction). (Lane 3) Total NCP extract Sephacryl 200 included volume (DGP-enriched fraction). (B) Autoradiographic exposure of gel electrophoresis of [^{125}I]-labeled dentin NCPs. NCPs separated by anti-DGP affinity chromatography. (Lane 4) Anti-DGP unbound fraction. (Lane 5) Anti-DGP bound fraction. (Lane 6) Purified DGP-γ_3. (Lane 7) Heterogeneous DGPs. NCPs had been separated prior to radioiodination by chromatography on a column of Sephacryl 200[14] (2.5 cm x 54 cm) eluted with 4 M guanidine/HCl[5], 50 mM Tris/HCl, pH 7.4, monitored at 280 nm.


A
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43K
30K
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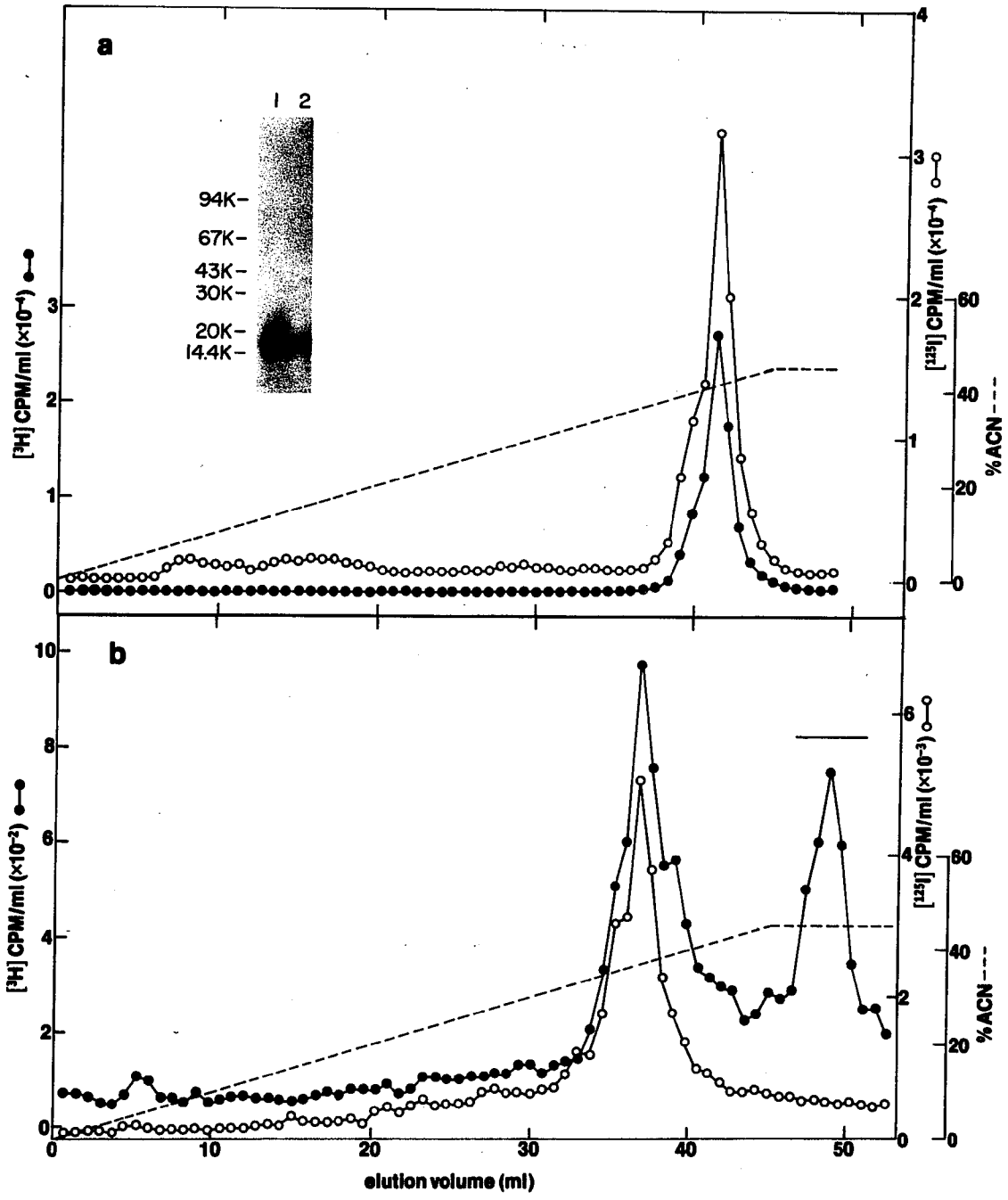
-94K
-67K
-43K
-30K
-20K
-14.4K
1 2 3 4 5 6 7

Fig. 4 - Reversed-phase high-performance liquid chromatography. Anti-DGP-bound proteins from formic acid extracts of 24 molars incubated with [^{3}H]-leucine (10 μCi/ml) for 24 hr at day 8 in vitro. (a) molars incubated with FCS. (b) molars incubated without FCS. Bar indicates second peak being characterized by SDS-PAGE/fluorography. (•) [^{3}H]-Leucine-labeled molar material; (o) [^{125}I]-adult rat DGP-γ_3. Inset, fluorogram exposed from SDS-polyacrylamide gel electrophoresis of [^{3}H]-leucine-labeled molar material isolated by reversed-phase HPLC. (Lane 1) Material from DGP peak in Fig. 4a. (Lane 2) Material from a DGP peak similar to that of 4b. Note: (a) and (b) represent runs that were several months apart; the difference in elution positions of [^{125}I]-DGP-γ_3 standard appears to be a minor chromatographic variation.


a
1 2
94K-
67K-
43K-
30K-
20K-
14.4K-
[3H] CPM/ml (×10-4)
[125I] CPM/ml (×10-4)
%ACN
b
[3H] CPM/ml (×10-2)
[125I] CPM/ml (×10-3)
%ACN
elution volume (ml)

authentic DGPs by SDS-PAGE. Another, yet unidentified, peak (bar) was present in Fig. 4b which was not seen in Fig. 4a or in a previous experiment with similarly prepared material (not shown). We suspect that this peak is additional DGP material, and it is currently being further characterized by SDS-PAGE/fluorography.

Immunolocalization. - In 11-day-old rat maxillary third molars cultured for ten days in mineralizing conditions (+ FCS), immunostaining was seen in odontoblasts and in dentin but not in predentin matrix or in any other cell type (Fig. 5A). Control sections incubated in a similar fashion with pre-immune serum did not stain, although a fine line of fluorescence could sometimes be seen at the dentino-enamel junction (Fig. 5B).

Molars cultured for ten days in non-mineralizing conditions (-FCS) showed immunostaining in odontoblasts (Fig. 5C). There was no staining in predentin or in any other cell type. Examination of the cervical loop region revealed immunoreactive odontoblasts in the area of the first appearance of predentin matrix; however, pre-odontoblasts in this region did not stain. Control sections also did not react (Fig. 5D).

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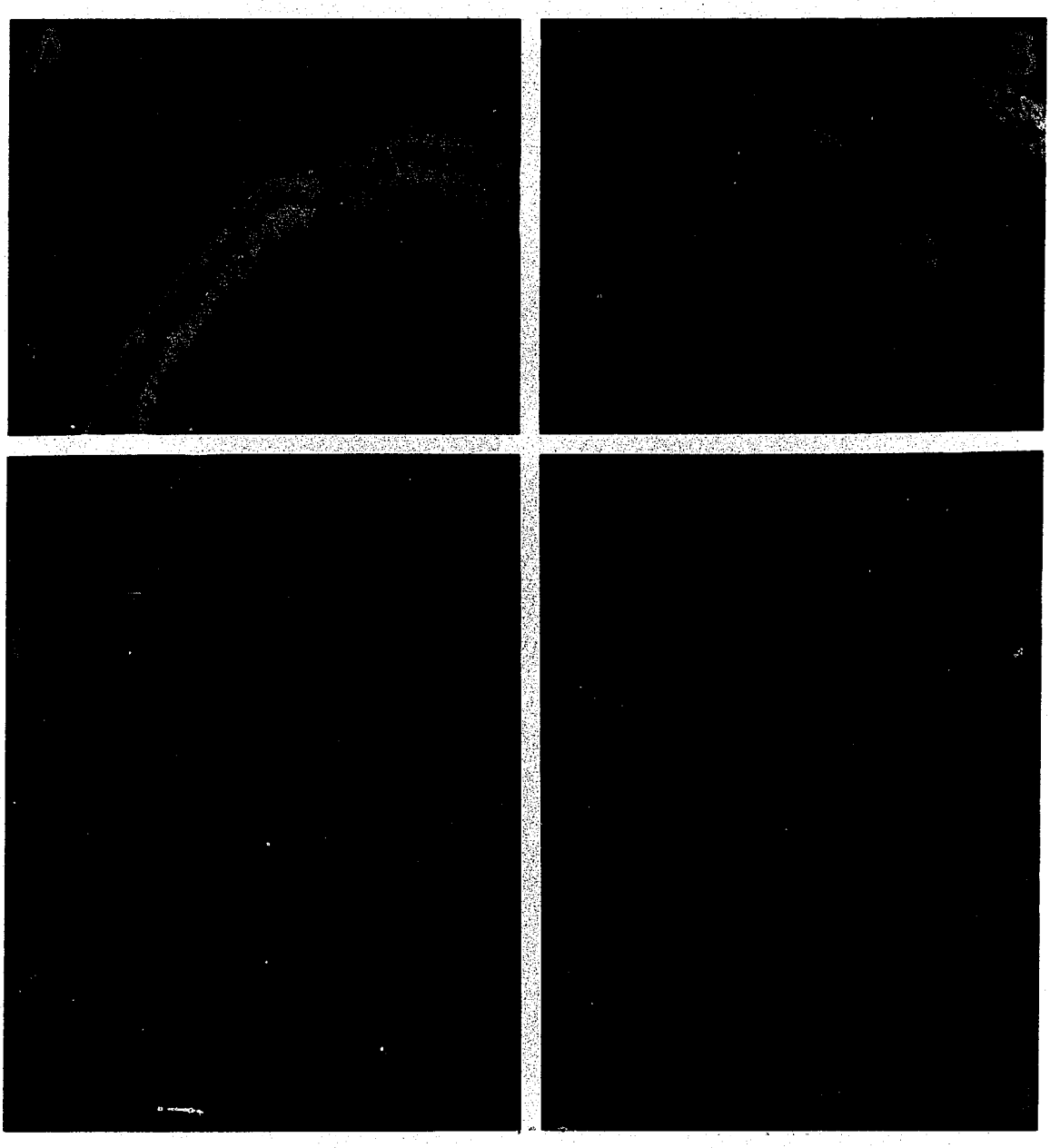
[28]Bio-Rad Laboratories, Richmond, CA

Fig. 5 - Immunolocalization of DGPs in molar organs by indirect immunofluorescence. Molars cultured for ten days (± FCS) were fixed, embedded, and sectioned. Sections were incubated with primary antibody (1:50 dilution), anti-DGP antiserum for experimental sections (A and E), or pre-immune serum for control sections (B and D), and then with secondary antibody, fluorescein isothiocyanate (FITC)-linked goat anti-rabbit immunoglobulins. Bars = 20 µm x 549.2. (A and B) Molars incubated with FCS. d, dentin; a, ameloblasts; pd, predentin; o, odontoblasts; p, pulp. (C and D) Molars incubated without FCS. pa, pre-ameloblasts.



DISCUSSION

The major objective of this study was a developmental one. The rat molar organ was used to characterize an *in vitro* model of mineralization, which was then used to correlate the *in vitro* biosynthesis of DGPs with the appearance of mineralized tissue.

This investigation has demonstrated that serum-supplemented medium permits *de novo* mineralization in molars *in vitro*, results that have been reported by many investigators (Wigglesworth, 1968; Thesleff, 1976; Bronckers *et al.*, 1983, Navia *et al.*, 1984). The increase in specific ^{45}Ca uptake after the sixth day in culture (Fig. 2A) corresponded to the first appearance of mineral at day 7 *in vitro* by histological evaluation (data not shown). Molars cultured in defined medium (-FCS) continued to develop and secrete predentin matrix in agreement with the results of Yamada *et al.* (1980), but at no time was there any evidence of mineral formation by biochemical or histological criteria (Figs. 2A, 1e, 1f).

Molars were taken from 11-day-old pups specifically to ensure that there were no mineralized tissues already present. It has been reported that the rat maxillary third molar starts to mineralize *in vivo* between 13 and 14 days of age (Schour and Massler, 1942) or between 12 and 13 days of age (Navia *et al.*, 1984). In separate experiments, mineralized dentin was occasionally seen in sections from molars removed from pups as young as 12 days, as indicated by positive Von Kossa staining (not shown). Since no mineral was present at 11 days of age (Fig. 1a, 1b), any mineralized tissue formed in the culture system reported here must have formed *de novo*.

HPLC had been used previously to show *in vitro* biosynthesis of DGPs by rat molars (Finkelman and Butler, 1984). This procedure has been

modified using an immunologic approach. Radiolabeled proteins extracted by formic acid from molars incubated with [^{3}H]-leucine in both mineralizing and non-mineralizing conditions (±FCS) were bound by the anti-DGP affinity column. Radioactivity in these bound fractions co-eluted with authentic adult rat incisor DGPs during reversed-phase HPLC and co-migrated with adult DGPs on SDS-PAGE (Fig. 4).

Rat dental cells synthesize DGPs *in vitro* (DiMuzio *et al.*, 1983; Finkelman and Butler, 1984). The present study has shown that rat molar odontoblasts, and no other cell types, are responsible for the biosynthesis of DGPs *in vitro*. A similar finding has been reported using *in vivo* molar material (Bronckers *et al.*, 1985). The observation of staining in dentin, but not in predentin, contributes to both sides of an interesting discussion regarding the extracellular processing of DGPs. Analysis of autoradiographic (Weinstock *et al.*, 1972; Weinstock and LeBlond, 1973), biosynthetic (DiMuzio and Veis, 1978) and preliminary immunohistochemical (MacDougall *et al.*, 1983) data has suggested that several dentin NCPs may be transported *via* odontoblastic processes through the predentin matrix and deposited directly at the mineralization front during dentin formation. The present immunohistochemical data may support such a transport process for DGPs. However, an alternate explanation is that DGPs are merely released from odontoblasts and are bound to dentin because of their Gla-dependent affinity for hydroxylapatite (Hauschka, 1981; Hauschka and Carr, 1982; Poser and Price, 1979), in a manner similar to the binding of BGP in bone (Price *et al.*, 1981). This idea is also supported by the observation reported herein, and by Bronckers *et al.* (1985), that predentin matrix will not

react immunohistochemically; it is only after mineralized dentin has formed that DGP antigens can be localized extracellularly.

It is of interest to note that molars cultured with FCS did not require demineralization to show immunostaining in dentin, in contrast to *in vivo* material in which dentin did not stain immunohistochemically without prior demineralization (Bronckers *et al.*, 1985). This observation may be due to a decreased density of the mineralized phase *in vitro*.

Analysis of the present data has suggested that rat odontoblasts synthesize DGPs in a manner that is not dependent on mineral but rather is concurrent with the elaboration of predentin matrix. The observation of immunostaining in odontoblasts, but not in pre-odontoblasts prior to predentin formation (seen in unmineralized molars, Fig. 5C), agrees with the results of Bronckers *et al* (1985). Similar findings were observed in mineralized molars (not shown).

From these data, one can see that DGPs are synthesized prior to the appearance of mineral. This finding appears to disagree with the observations that the deposition of BGP occurs after the onset of mineralization in developing human and rat bone (Price *et al.*, 1981) and that BGP appears coincidently with mineralization in chick bone (Hauschka *et al.*, 1983). However, our investigations are studies related to the biosynthesis of DGPs, whereas the studies mentioned above focused on the deposition and accumulation of BGP. Thus, since biosynthesis and deposition represent different physiological events, the above observations are not necessarily in conflict. The immunohistochemical data reported here do indeed support the notion that the

accumulation of Gla-containing proteins is dependent on the presence of hydroxylapatite.

The present investigation raises further questions regarding the synthesis and secretion of DGPs. Is the biosynthesis of DGPs quantitatively similar in both the mineralizing and non-mineralizing molar? Are DGPs synthesized by non-mineralizing molars degraded, or are they released into the media? Further studies are required to address these questions.

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APPENDIX 1

DETAIL OF MOLAR ORGAN CULTURE[1]

Materials and Methods

Animals

Timed-pregnant female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA). Immediately upon arrival at the University of Alabama at Birmingham, animals were housed individually in plastic cages and fed purified diet MIT 305 (284) (Tables 1-3) and distilled water ad libitum. They were housed in a room with a controlled alternating light/dark cycle (12 hours light/12 hours dark). Cages were changed weekly, and animals were given clean cages the day before they were expected to deliver. Within 24 hours after delivery, all litters were reduced in size to eight, with pups randomized among the dams. Pups were managed such that after being weighed, the pups of least and greatest weight were sacrificed. Pups of intermediate weight were utilized for the experiment. Dams were rotated daily among the litters in a sequential fashion so that all litters received approximately the same nutritional support.

Medium

A detail of the medium used has been presented (117). The formulation of Eagle's minimum essential medium is presented in Table 4.

Dissection

As previously mentioned, all rat pups used were 11 days of age. The neck was disinfected with iodine solution (Merthiolate, Sigma Chemical Co., St. Louis, MO), and pups were decapitated by guillotine.

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[1] modified from Navia, et al. (283)

TABLE 1

Composition of diet MIT 305[1]

component	percent of total (w/w)
sucrose	5.0
cornstarch	62.0
lactalbumin	20.0
cottonseed oil	3.0
cellulose	6.0
vitamin mix (Table 2)	1.0
salt mix (Table 3)	3.0
	100.0

[1]from Navia (284)

TABLE 2

Composition of vitamin mixture for diet MIT 305

component	percent of total (w/w)
thiamine (B_1)	0.114
riboflavin (B_2)	0.086
pyridoxine (HCl) (B_6)	0.043
cyanocobalamine (0.1% mannitol) (B_{12})	0.429
niacin (nicotinic acid)	0.286
Ca pantothenate	0.429
para-aminobenzoic acid (PABA)	2.857
inositol	28.571
biotin	0.001
menadione (vitamin K)	0.043
folic acid	0.029
ascorbic acid (vitamin C)	1.429
retinol (vitamin A)	0.871
α-tocopherol (vitamin E)	3.571
cholecalciferol (vitamin D_3)	0.087
cellulose	61.540
	100.000

TABLE 3

Composition of salt mixture for diet MIT 305

component	percent (w/w)
$CaHPO_4$	49.1060
KCl	14.1860
NaCl	23.2851
K_2SO_4	6.1830
$MgSO_4$	1.9820
$MgCO_3[4MgCO_3 \cdot (OH)_2 \cdot 5H_2O]$	2.6270
ferric citrate	1.8000
$MnCO_3$	0.3630
$CuCo_3$	0.0529
$ZnCO_3$	0.2700
KIO_3	0.0051
$AlCl_3 \cdot 6H_2O$	0.0295
$CoCl_2 \cdot 6H_2O$	0.0094
cellulose	0.1010
	100.0000

TABLE 4

Formulation of Eagle's minimum essential medium [1]

component	amount (mg/l)
Inorganic salts:	
$CaCl_2$ (anhydrous)	200.00
KCl	400.00
$MgSO_4$ (anhydrous)	97.72
NaCl	6800.00
$NaH_2PO_4 \cdot H_2O$	140.00
Other Components:	
glucose	1000.00
phenol red	10.00
Amino Acids:	
L-arginine•HCl	126.00
L-cystine•2HCl	31.29
L-glutamine	292.00
L-histidine HCl•$2H_2O$	42.00
L-isoleucine	52.00
L-leucine	52.00
L-lysine•HCl	72.50
L-methionine	15.00
L-phenylalanine	32.00
L-threonine	48.00
L-tryptophane	10.00
L-tyrosine (disodium salt)	52.10
L-valine	46.00
Vitamins:	
D-Ca-pantothenate	1.00
choline chloride	1.00
folic acid	1.00
i-inositol	2.00
nicotinamide	1.00
pyridoxal HCl	1.00
riboflavin	0.10
thiamine HCl	1.00

[1] Tissue Culture Products Catalogue (1982), GIBCO Laboratories, Grand Island, NY

All subsequent procedures were performed aseptically in a laminar-flow hood (EdgeGARD Hood, Baker Co., Inc., Sanford, ME). After being skinned and the mandible removed, the head was dipped in 70% ethyl alcohol for 10 seconds. The third molar was uncovered under a stereo-microscope (M5, Wild Heerbrugg, Switzerland) by carefully removing overlying palatal mucosa and bone. The third molar was removed as gently as possible with a dental instrument, rinsed with an antibiotic-antimycotic (penicillin/streptomycin/fungizone, GIBCO, Grand Island, NY) and cultured as described (117).

Results and Discussion

Use of 11-Day-Old Rats

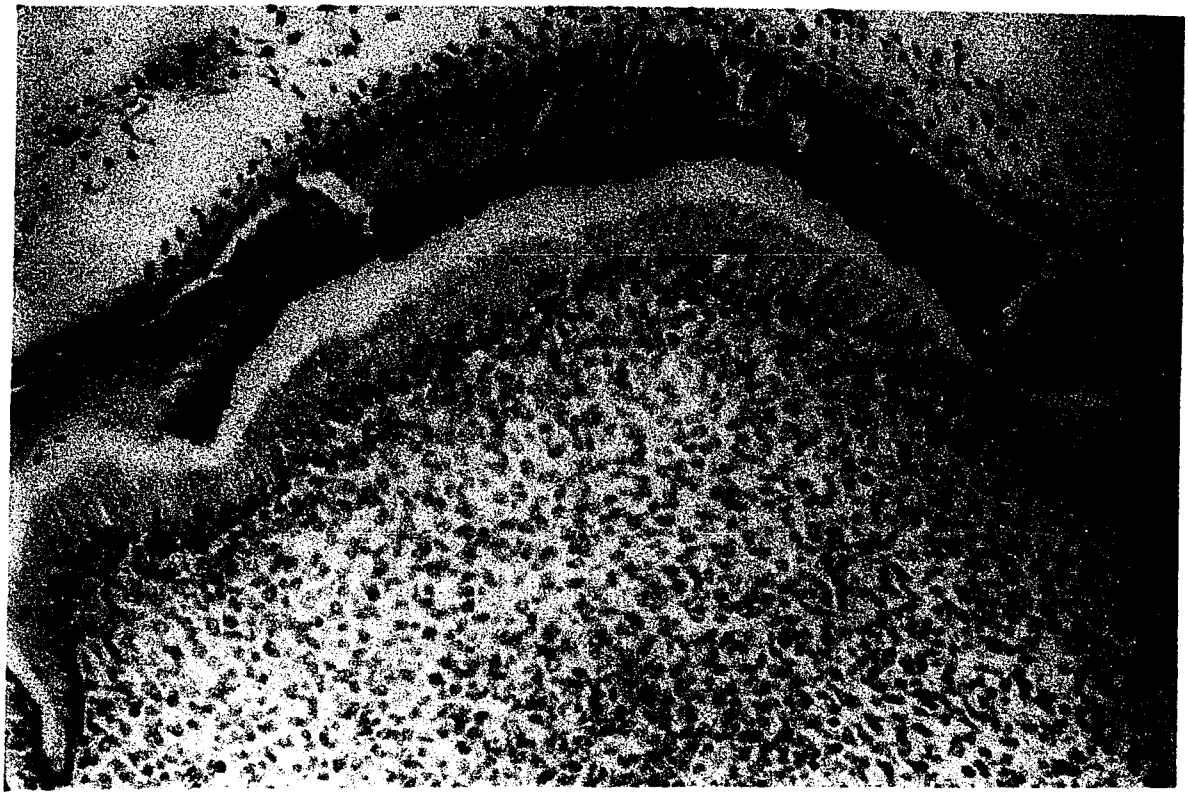
Maxillary third molars *in vivo* were examined histologically at various days of age. Third molars from 15-day-old rats had developed so that mineralized dentin and enamel were abundant (Figure 2). In third molars from 12-day-old rats, pre-ameloblasts, odontoblasts and predentin were present. Occasionally, mineralized dentin was present (Figure 3). Third molars from 11-day-old rats showed no evidence for the presence of mineralized dentin (117). Thus, although maxillary third molars in general have not started to mineralize *in vivo* by 12 days of age, use of molars from 11-day-old rats insured that absolutely no mineralized dentin was present at the start of the experiment.

Dissection and Culture

Figures 4-7 present dissection and culture as visualized by stereo-microscopy. The palate of the 11-day-old rat was examined, and the molar teeth were exposed (Figure 4). At this stage of development the third molar was a roughly spherical organ that could be freed from its attachment and removed by a dental instrument (Figure 5, top). In

Figure 2. Maxillary third molar from 15-day-old rat stained with Von Kossa/nuclear fast red. x 172.6.

Figure 3. Maxillary third molar from 12-day-old rat stained with Von Kossa/nuclear fast red. Top: x 176.0; bottom: x 442.9.



contrast, crown development of the maxillary second molar was just about complete at 12 days of age (Figure 5, bottom). The third molar germ was placed in culture supported on a filter (Figure 6, top). Molar growth and cuspal development was evident in defined medium and in serum-supplemented medium (Figure 6, middle and bottom). Mineral was first evident histologically in molars cultured in serum-supplemented medium at the seventh day in vitro (Figure 7, top) and was more abundant by the tenth day (Figure 7, bottom).

18-Day Molar Culture

In consideration of the 10-day ^{45}Ca-uptake experiment presented elsewhere (Figure 2 [117]), there was a possibility that molars cultured without serum would still mineralize if cultured for a longer period. Molars cultured in defined medium showed a slight increase in ^{45}Ca-uptake at the tenth day. Would ^{45}Ca-uptake rise significantly after the tenth day, or would it remain fairly constant (or even fall)? In other words, if ^{45}Ca-uptake is considered as a measure of mineralization, was mineralization in molars cultured without serum merely delayed relative to those cultured with serum supplementation, or would those molars indeed not mineralize at all?

Results of an experiment designed to address this question are presented in Figure 8. Molars were cultured for periods up to 18 days. At four-day intervals, groups of molars were incubated with ^{45}Ca as described previously. At the end of the incubation period, molars were harvested and treated as described. Results indicated that molars cultured without serum showed a trend of decreasing ^{45}Ca-uptake after the tenth day, although the change was not significant. In contrast, molars cultured with serum continued to maintain a significantly

Figure 4. 11-day-old rat. Top: palate showing posterior alveolar ridges. x 17.8; middle: maxillary alveolar ridge with overlying mucosa and bone removed revealing first, second and third molars. x 35.7; bottom: maxillary third molar *in situ*. x 72.9.

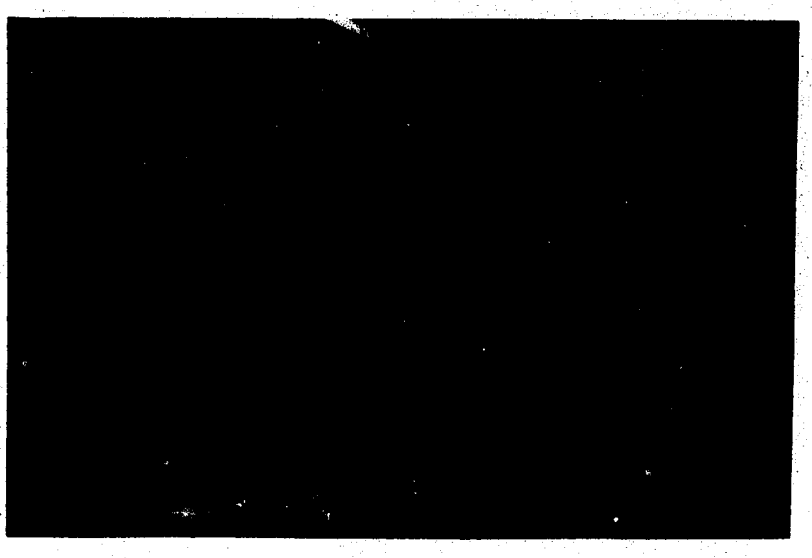


Figure 5. Maxillary teeth from 11-day-old rat on dental instrument. Top: third molar. x 110; bottom: second molar. x 53.5.

Figure 6. Maxillary third molars from 11-day-old rat in organ culture. Top: at time of explant. x 72.9; middle: after 8-day incubation in serum-free medium. x 33.6; bottom: after 10-day incubation in serum-supplemented medium. x 70.7.

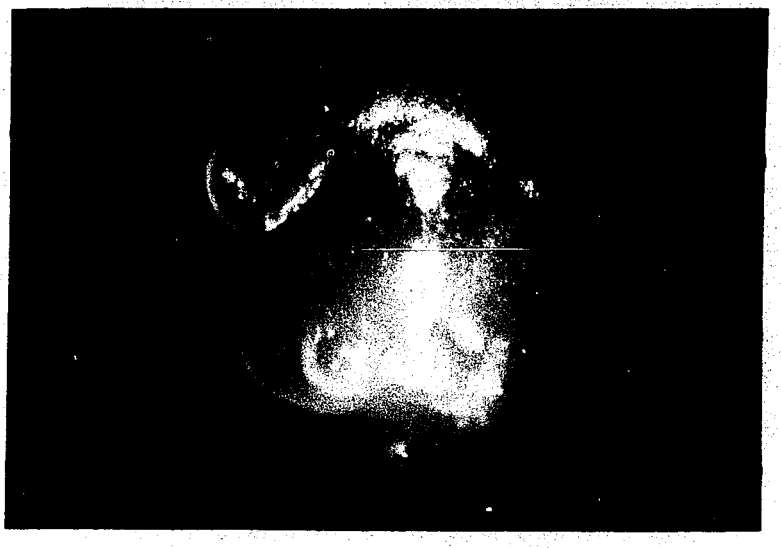
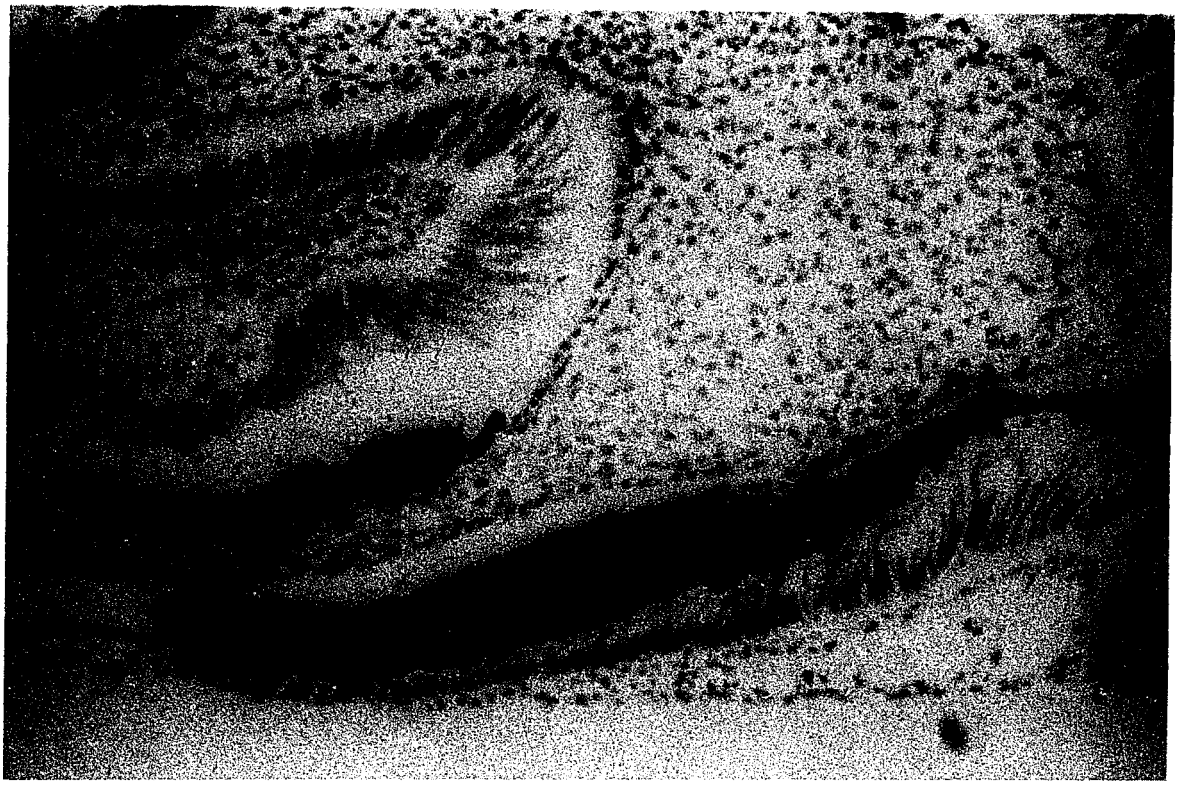


Figure 7. Maxillary third molars from 11-day-old rat cultured in serum-supplemented medium stained with Von Kossa/nuclear fast red. x 440. Top: 7-day incubation; bottom: 10-day incubation.



elevated uptake of radiolabel after day 10. These data suggest that serum-supplemented molars continued to form mineralized dentin through the 18th day of the experiment, but those molars cultured in defined medium did not form mineralized dentin. Statistical evaluations by ANOVA and multiple means comparisons are presented in Table 5.

Conclusions

Molars cultured without serum did not show evidence of mineralization for an 18-day culture period. Molars cultured with serum showed evidence for continued mineralization after the sixth day of an 18-day culture period. Molars in both conditions apparently were viable and showed cusps after 10 days *in vitro*.

Figure 8. Parameters of *in vitro* molar development, 18 days. Molars were incubated (± FCS) with ^{45}Ca (5 µCi/ml) for 24 hours at the times specified. Molars were lyophilized, weighed and extracted for 24 hours with 10% formic acid. Each point represents the average of 8 molars ± S.D. (a) ^{45}Ca-uptake per µg per molar per day *in vitro*; (b) dry weight per molar per day *in vitro*. (•) Molars cultured with FCS; (o) molars cultured without FCS. * = significantly different from - FCS group, $p < 0.05$. ** = significantly different from - FCS group, $p < 0.001$.

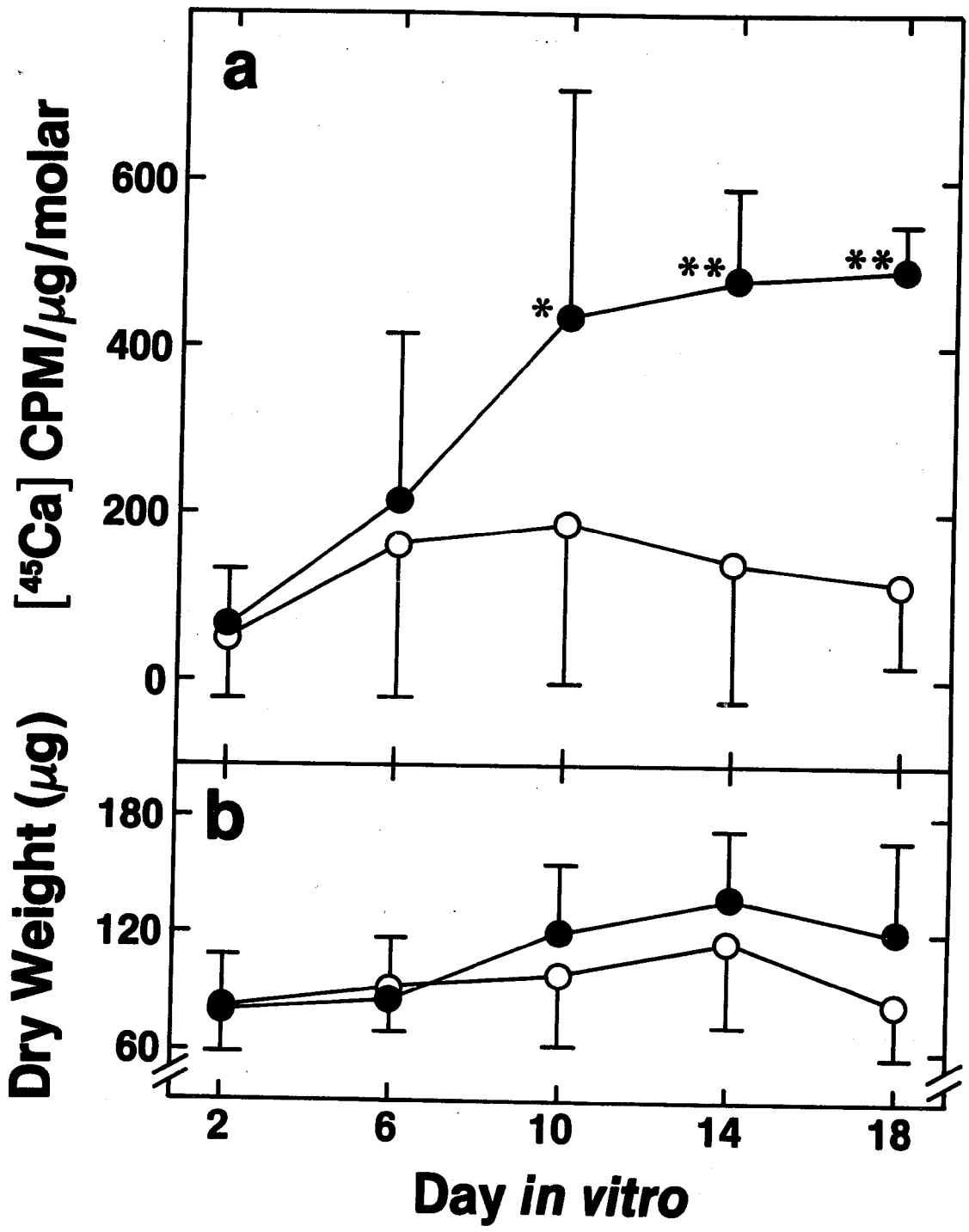

a
b
600
400
200
0
180
120
60
*
**
**
2
6
10
14
18
[45Ca] CPM/μg/molar
Dry Weight (μg)
Day in vitro

TABLE 5

Statistical evaluation of *in vitro* molar development, 18 days

Dry Weight, - FCS

ANOVA Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Between days	5,697.4888	4	1,424.3723	1.3198, n.s.[1]
Within days	36,693.5448	34	1,079.2219	

Dry Weight, + FCS

ANOVA Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Between days	20,074.2307	4	5,018.5577	4.4645, $p < 0.01$
Within days	38,219.7083	34	1,124.1091	

multiple means comparison[2]

Day	2	6	10	18	14
Dry weight	79.93	86.69	121.31	124.56	137.36

[1] n.s. = not significant

[2] Underlining indicates groups not significantly different. Any means not joined by same underline are significantly different ($p < 0.05$).

TABLE 5 (continued)

^{45}Ca-Uptake, - FCS

ANOVA Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Between days	87,674.6535	4	21,918.6634	0.9644, n.s.
Within days	772,716.9155	34	22,726.9681	

^{45}Ca-Uptake, + FCS

ANOVA Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Between days	992,398.931	4	248,099.7328	7.5352, p<0.01
Within days	1,119,465.984	34	32,925.4701	

multiple means comparison

Day	2	6	10	14	18
Uptake	66.4	215.8	437.9	475.7	495.0

APPENDIX 2

ANTIBODY CHARACTERIZATION

As previously described (117), a rabbit anti-rat polyclonal antibody was raised against a highly purified dentin Gla fraction, dentin Gla-γ_3. An affinity column prepared with this antibody will selectively bind DGPs (117). Previous work in this laboratory had suggested that anti-Gla antibodies may cross-react (albeit weakly) with dentin 95K (285). In order to detect any possible cross-reactivity with greater sensitivity, anti-DGP-γ_3 was further characterized by RIA.

Methods

The RIA presented herein was modified from Price, et al. (18). All assays contained (in order of addition): (1) a known amount of unlabeled protein in 0.1 ml of incubation buffer (Table 6); (2) rabbit anti-serum in 0.1 ml of incubation buffer (final dilution of 1:1600); (3) 0.1 ml of incubation buffer; and (4) [^{125}I]-DGP-γ_3 (approximately 20,000 CPM) in 0.1 ml of incubation buffer. All components except radiolabel were combined and incubated for one hour at 37°C with shaking. Labeled DGP-γ_3 was added and assay mixtures were incubated again for one hour at 37°C. Assays were terminated by addition of Staphylococcus aureus (507861, Calbiochem-Behring, La Jolla, CA) in 0.1 ml of incubation buffer. After 30 minutes at 37°C with shaking, mixtures were centrifuged and the supernatants discarded. Pellets were washed with one ml of wash buffer (Table 7) and centrifuged again. Wash procedures were repeated twice and final pellets were counted for ^{125}I activity.

Results and Discussion

The inhibition RIA is shown in Figure 9. A preparation of non-collagenous proteins separated by chromatography on a column of

TABLE 6

RIA incubation buffer

component	concentration	amount
sodium deoxycholate	0.5 %	5 g/l
bovine serum albumin	0.1 %	1 g/l
Nonidet P-40	0.1 %	1 ml/l

Preparation: diluted in PBS

TABLE 7

RIA wash buffer

component	dilution	amount
NaCl	0.154 M	9.00 g/l
Tris	0.010 M	1.21 g/l
NaN_3	0.03 %	0.30 g/l
sodium deoxycholate	0.25 %	2.50 g/l
Nonidet P-40	0.25 %	2.00 ml/l

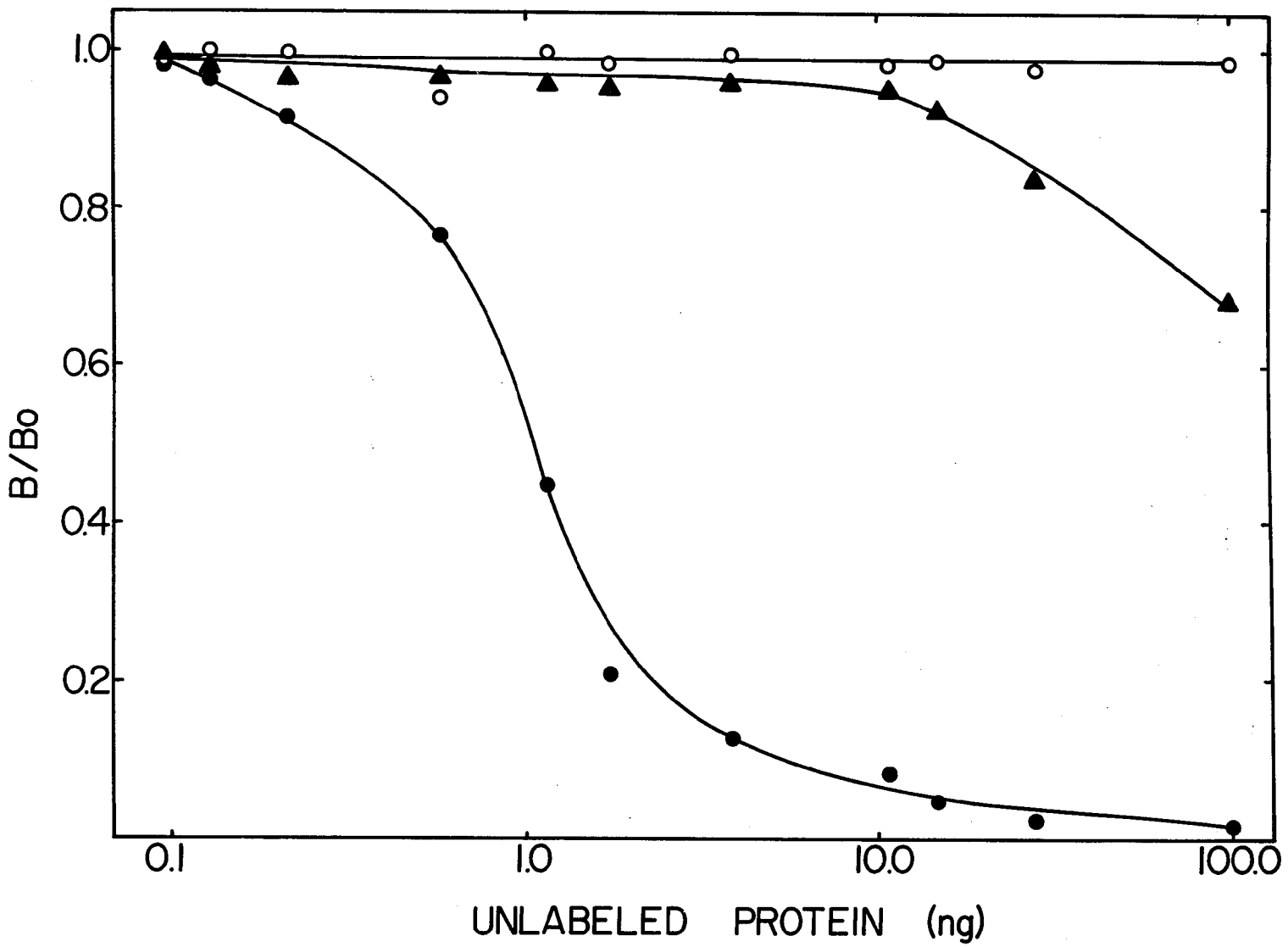
Preparation: diluted in PBS

Sephacryl 200 (Figure 3 [117]) was used for this assay. This preparation was devoid of the Mr = 14,000-18,000 dentin Gla proteins. Figure 9 shows that anti-DGP-γ_3 antibody did not cross-react with any other dentin non-collagenous proteins (including 95K) in the dilutions measured and bound DGP-γ_3 with a half-maximal value at a concentration of 1 ng/400 µl. Anti-DGP-γ_3 displayed some cross-reactivity with a dentin non-collagenous protein preparation termed Fraction III (8), a fraction highly enriched in 95K. Since anti-DGP-γ_3 did not cross-react with the Sephacryl 200 void preparation (which contained 95K), this cross-reactivity with Fraction III was interpreted to be due to a contamination of the Fraction III preparation with some DGP material.

Conclusion

Anti-DGP-γ_3 antibody is highly specific and is useful for studies regarding DGPs.

Figure 9. Competitive-binding assay of dentin non-collagenous proteins using anti-DGP-γ_3 antibody. Known amounts of unlabeled protein, anti-DGP-γ_3 antibody and [^{125}I]-DGP-γ_3 were incubated together as described in Methods. (•) DGP-γ_3; (Δ) dentin Fraction III; (o) Sephacryl 200-void fraction (DGP-depleted) of dentin NCPs.


1.0
0.8
0.6
0.4
0.2
B/Bo
0.1
1.0
10.0
100.0
UNLABELED PROTEIN (ng)

APPENDIX 3

PROTEIN BIOSYNTHESIS IN VITRO

Since molars incubated with serum mineralized and those incubated without serum did not, an obvious question was raised as to whether there were any differences in protein biosynthesis between the two groups of molars. Additionally, information was needed concerning protein biosynthesis over a 10-day culture period.

Methods

Total Proteins

Molars cultured with or without serum (± FCS) were incubated with [^{3}H]-leucine (10 µCi/ml) for 24 hours at the eighth day of culture. Molars were then incubated for one hour in fresh, unlabeled medium before the cultures were terminated. Tooth germs were pooled and sonicated (Branson Sonicator, microprobe setting no. 6) on ice in 4 M Gdm-Cl, 0.2 M EDTA and 20 mM Tris-HCl (pH 7.4) containing protease inhibitors (109). Extraction was continued overnight at 4°C with shaking. After being centrifuged, the solution was desalted on a column of Sephadex G-25 (fine, 10 cm x 0.9 cm) eluted with 10 mM sodium phosphate (pH 7.0) with protease inhibitors at 4° C in order to remove unincorporated counts. Only fractions containing macromolecular counts were utilized for further analysis. Total protein extracts were electrophoresed and analyzed by fluorography as described (117).

Protein Biosynthesis

Groups of molars cultured ± FCS were incubated with [^{3}H]-leucine (10 µCi/ml) and $^{45}CaCl_2$ (5 µCi/ml) for 24 hours at various intervals of a 10-day culture period. At the end of the incubation period, molars were washed three times with unlabeled medium, lyophilized and weighed.

Groups of molars were extracted overnight with 10% formic acid at 4°C with shaking. Aliquots were counted for ^{3}H and ^{45}Ca activity, and the remaining extracts were chromatographed on a column (Sephadex G-25 [fine, 10 cm x 0.9 cm, 0.2 M NH_4HCO_3]) to separate macromolecular from unincorporated counts. Aliquots of the void peak were counted to determine macromolecular incorporation of ^{3}H. Molar residues were then extracted with 4 M Gdm-Cl, 20 mM Tris-HCl (pH 7.4) with protease inhibitors. Extracts were desalted as above, and aliquots of the void fraction were counted to measure ^{3}H incorporation into Gdm-Cl-extractable proteins. Macromolecular ^{3}H activities in both formic and Gdm-Cl extracts were added to give a measure of total protein biosynthesis.

Results and Discussion

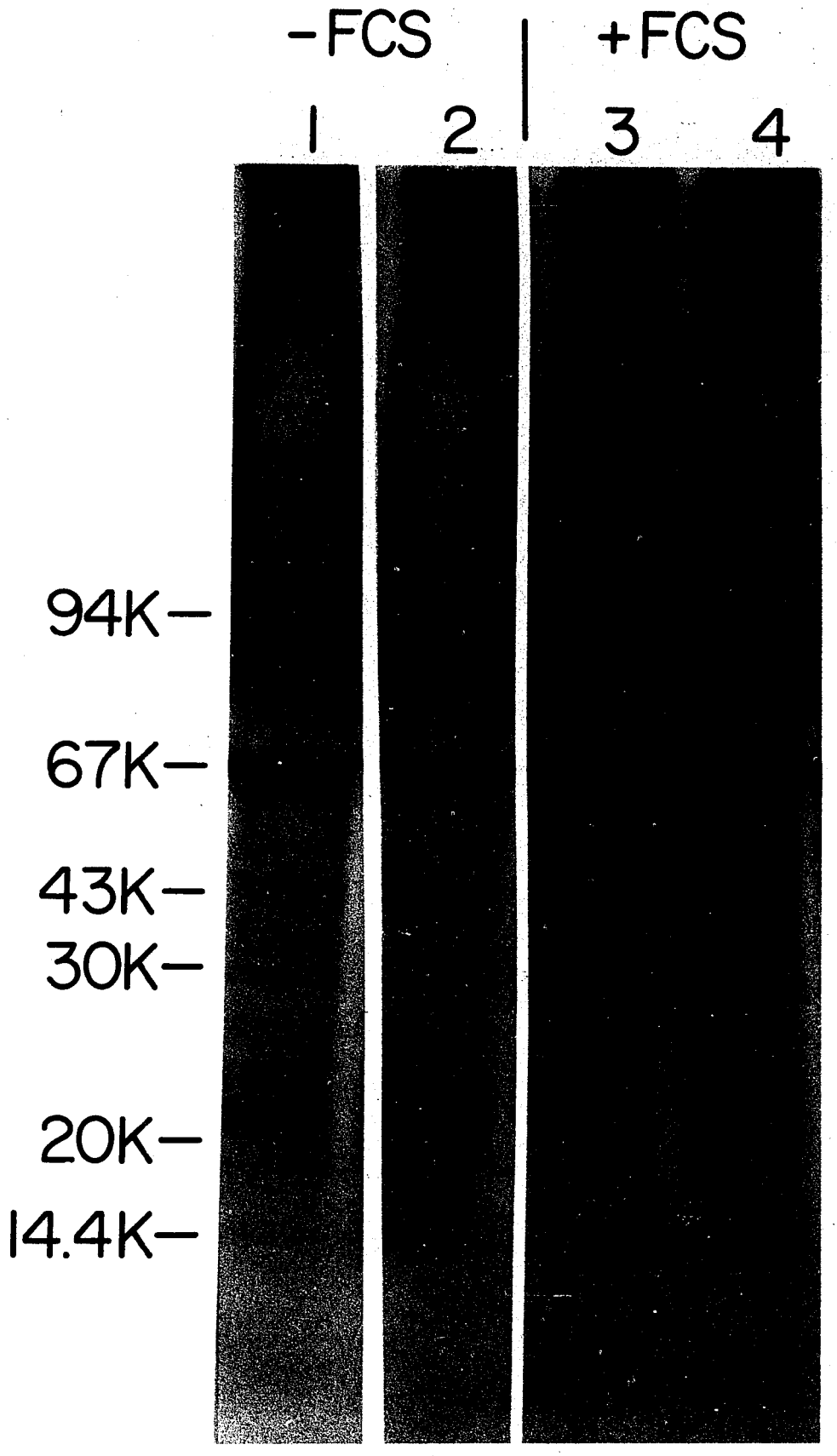
Total Protein Synthesis

When analyzed by SDS-PAGE, molars from both groups (± FCS) synthesized similar protein components (Figure 10). There appeared to be quantitative but not qualitative differences. One must use caution in interpreting these data, however, since cell types other than odontoblasts and ameloblasts may dominate in the molar organ. Dentin- and enamel-specific proteins might be relatively minor components of the total protein profile.

Protein Biosynthesis

In agreement with previous results (117), molars cultured without FCS did not show a significant increase in ^{45}Ca-uptake for a 10-day culture period, while molars cultured with FCS showed a significant increase in ^{45}Ca-uptake after the sixth day *in vitro* (Figure 11a). Different results were obtained, however, from analysis of uptake of ^{3}H. Molars from both groups (± FCS) showed a general trend of increasing

Figure 10. Fluorogram, SDS-polyacrylamide gel electrophoresis of total molar proteins. Proteins extracted in 4 M Gdm-HCl, 0.2 M EDTA and 20 mM Tris/HCl (pH 7.4) containing protease inhibitors. Molars incubated (± FCS) with [^{3}H] (10 µCi/ml) at day 8 *in vitro* for 24 hours. Lanes 1 and 2, - FCS; lanes 3 and 4, + FCS.


-FCS
+FCS
1
2
3
4
94K—
67K—
43K—
30K—
20K—
14.4K—

protein biosynthesis over the course of the experimental period, although biosynthesis began to decrease in the - FCS group at the tenth day (Figure 11a). Molars from both groups (± FCS) showed significant variation in molar dry weight, but there was no significant difference between the two groups at any day (Figure 11b). Statistical evaluation of these data is presented in Table 8.

Conclusions

Molars cultured with and without serum synthesize qualitatively similar proteins and show a general trend of an increasing rate of protein biosynthesis over a 10-day culture period. Quantitative differences were noted in the biosynthesis of several protein components by molars incubated in mineralizing and non-mineralizing conditions.

Figure 11. Parameters of *in vitro* molar development, 10 days. Molars were incubated (± FCS) with $^{45}CaCl_2$ (5 µCi/ml) and with [^{3}H]-leucine (10 µCi/ml) for 24 hours at the times specified. Molars were lyophilized, weighed and extracted successively with 10% formic acid and then with 4 M Gdm-HCl, 20 mM Tris/HCl (pH 7.4) containing protease inhibitors. Each point represents either the total of 8 molars or the average of 8 molars ± S.D. when S.D. indicated. (a) ^{45}Ca- and ^{3}H-uptake per day *in vitro*; (b) dry weight per molar per day *in vitro*. (•) + FCS; (o) - FCS. * = significantly different from - FCS group, $p < 0.05$; ** = significantly different from - FCS group, $p < 0.001$.

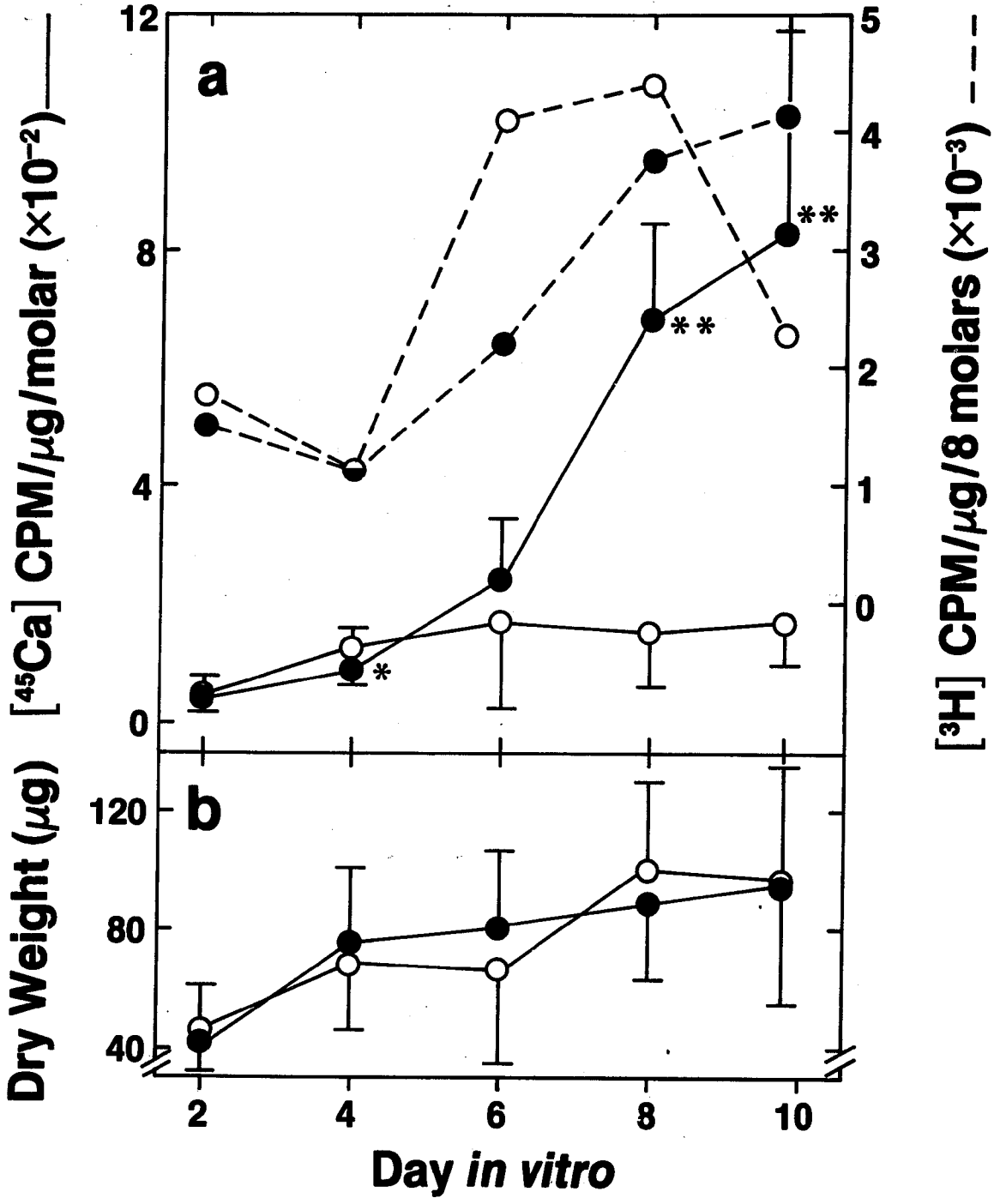

a
b
12
8
4
0
5
4
3
2
1
0
120
80
40
2
4
6
8
10
*
**
**
Dry Weight (μg) [45Ca] CPM/μg/molar (×10−2)
[3H] CPM/μg/8 molars (×10−3)
Day in vitro

TABLE 8

Statistical evaluation of *in vitro* molar development, 10 days

Dry Weight, - FCS

ANOVA Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Between days	20,376.85	4	5,094.2125	5.9982, $p<0.01$
Within days	29,725.125	35	849.2893	

multiple means comparison

Day	2	6	4	8	10
Weight	46.1	66.8	67.9	100.0	106.6

Dry weight, + FCS

ANOVA Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Between days	16,928.250	4	4,232.0625	6.488, $p<0.01$
Within days	22,830.125	35	652.2893	

multiple means comparison

Day	2	4	6	8	10
Weight	41.8	75.1	80.9	87.1	104.5

TABLE 8 (continued)

^{45}Ca-Uptake, - FCS

ANOVA Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Between days	80,334.9715	4	20,083.7429	2,6342, n.s.
Within days	266,847.1325	35	7,624.2038	

^{45}Ca-Uptake, + FCS

ANOVA Table

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Between days	4,105,015.333	4	1,026,253.833	36.1603, p<0.01
Within days	993,324.134	35	38,380.6895	

multiple means comparison

Day	2	4	6	8	10
Uptake	41.2	89.4	241.6	683.8	830.0

APPENDIX 4

BIOSYNTHESIS OF DENTIN GLA-CONTAINING PROTEINS

A slightly different technique was utilized initially to identify DGP biosynthesis *in vitro*. This technique took advantage of the lower Mr of DGPs. In addition, it was desirable to gain some information regarding both the variation of DGP biosynthesis over a 10-day experiment and an approximate value as to the fraction of total protein synthesis represented by DGPs.

Methods

Evidence for DGP Biosynthesis

Molars were incubated with [^{3}H]-leucine (10 µCi/ml) for 24 hours in serum-supplemented medium at the seventh day of culture. After the labeling period, molars were incubated with fresh, unlabeled medium for one hour. Molars were pooled, sonicated on ice and extracted for 24 hours in 4 M Gdm-Cl, 0.2 M EDTA and 20 mM Tris-HCl (pH 7.4) containing protease inhibitors. Extracts were separated (Sephadex G-25 [fine, 10 cm x 0.9 cm], 10 mM sodium phosphate [pH 7.0] with protease inhibitors), and macromolecular fractions were applied to an HPLC TSK G-3000 molecular sieve column (2 x [7.5 mm x 60 cm]) eluted with 2 M Gdm-Cl, 10 mM sodium phosphate, pH 7.0. [^{125}I]-Labeled adult rat incisor DGPs were chromatographed in an identical fashion. Appropriate fractions of the molar samples from gel filtration were pooled, desalted (Sephadex G-25 [fine, 10 cm x 0.9 cm], 0.2 M NH_4HCO_3) and applied to an HPLC reversed-phase µBondapak C-18 column (3.9 mm x 30 cm), eluted with 1% TFA and a gradient of 0-45% acetonitrile (ACN). [^{125}I]-DGP standard was chromatographed in an identical fashion.

Variation in DGP Synthesis

Groups of molars (Appendix 3) were incubated (± FCS) with [^{3}H]-leucine (10 µCi/ml) and $^{45}CaCl_2$ (5 µCi/ml) for 24 hours at various times of a 10-day culture period. Molars were extracted and radioactivity measured as described (Appendix 3). Formic acid-extractable proteins were separated by affinity chromatography (anti-DGP-γ_3 [117]). Radioactivity in bound fractions was determined and computed as a fraction of total [^{3}H]-labeled protein.

Results and Discussion

Identification of DGPs

Figure 12 (top) shows that a minor peak from the molar sample co-eluted with [^{125}I]-labeled DGP standard on gel filtration chromatography. [^{3}H]-Labeled material from this peak also co-eluted with DGP standard on reversed-phase chromatography (Figure 12, bottom). This chromatographic combination provided convincing evidence for the identification of this molar-derived material as DGPs, since the sample displayed Mr and hydrophobic characteristics identical to DGP standard.

DGP Uptake of Radiolabel

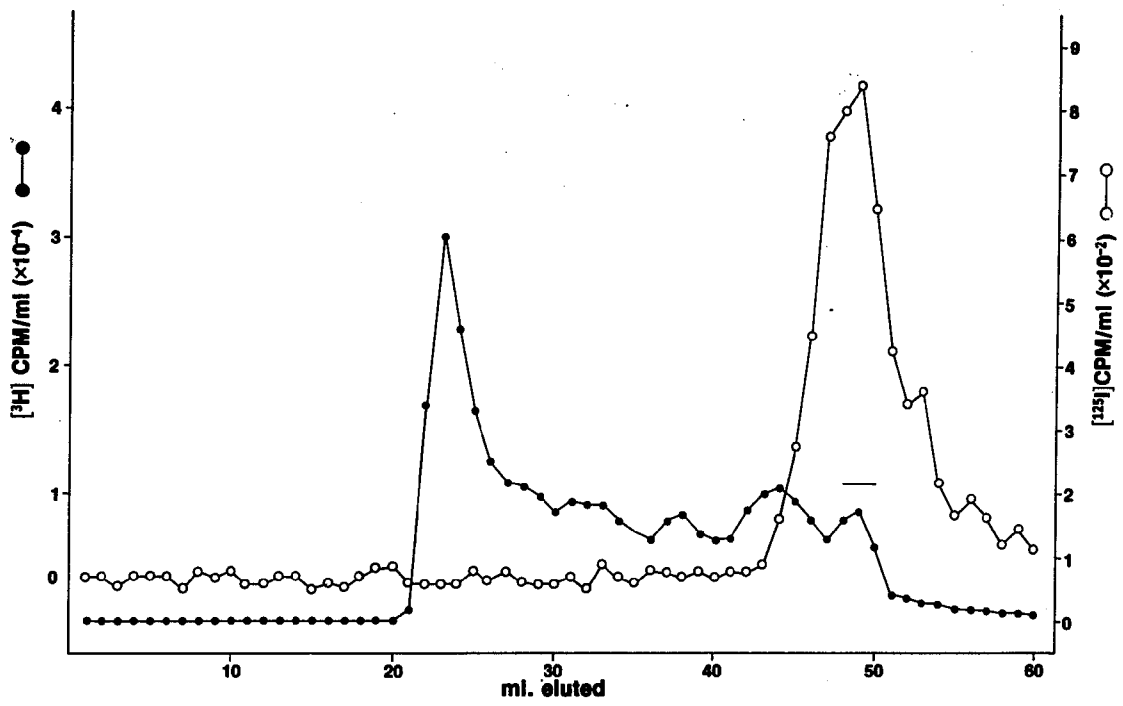
These data are presented in Table 9 and in Figure 11a. DGP biosynthesis was independent of mineralization (^{45}Ca-uptake). DGP biosynthesis paralleled the general trend of increasing total protein biosynthesis, and uptake of radiolabel into anti-DGP-γ_3-bound material represented 0.5-1.0% of radiolabeled acid- and Gdm-Cl-extractable protein.

Conclusions

Molars synthesize DGPs <u>in vitro</u> independently of mineralization. Molars show a general trend of an increasing rate of biosynthesis of

DGPs for a 10-day culture period, in a manner paralleling total protein synthesis.

Figure 12. HPLC identification of dentin Gla-containing proteins. Top: gel filtration. Total tooth germ sonicate and [^{125}I]-labeled adult rat incisor Gla-containing proteins were chromatographed on a TSK G-3000 column [2 x (7.5 mm x 60 cm)], eluted with 2 M Gdm-HCl, 10 mM sodium phosphate (pH 7.0). Tooth germs were incubated with [^{3}H]-leucine (10 µCi/ml) at the seventh day in vitro for 24 hours. (-) indicates region taken for reversed-phase HPLC. Bottom: reversed phase. DGP-containing fractions from Figure 12 (top) and [^{125}I]-labeled adult DGPs were chromatographed on a µBondapak C-18 column (3.9 mm x 30 cm), eluted with 1% TFA and a gradient of 0-45% ACN. (•) molar sample; (o) [^{125}I]-labeled adult rat DGPs.


[3H] CPM/ml (×10⁻⁴)
[125I]CPM/ml (×10⁻²)
ml. eluted

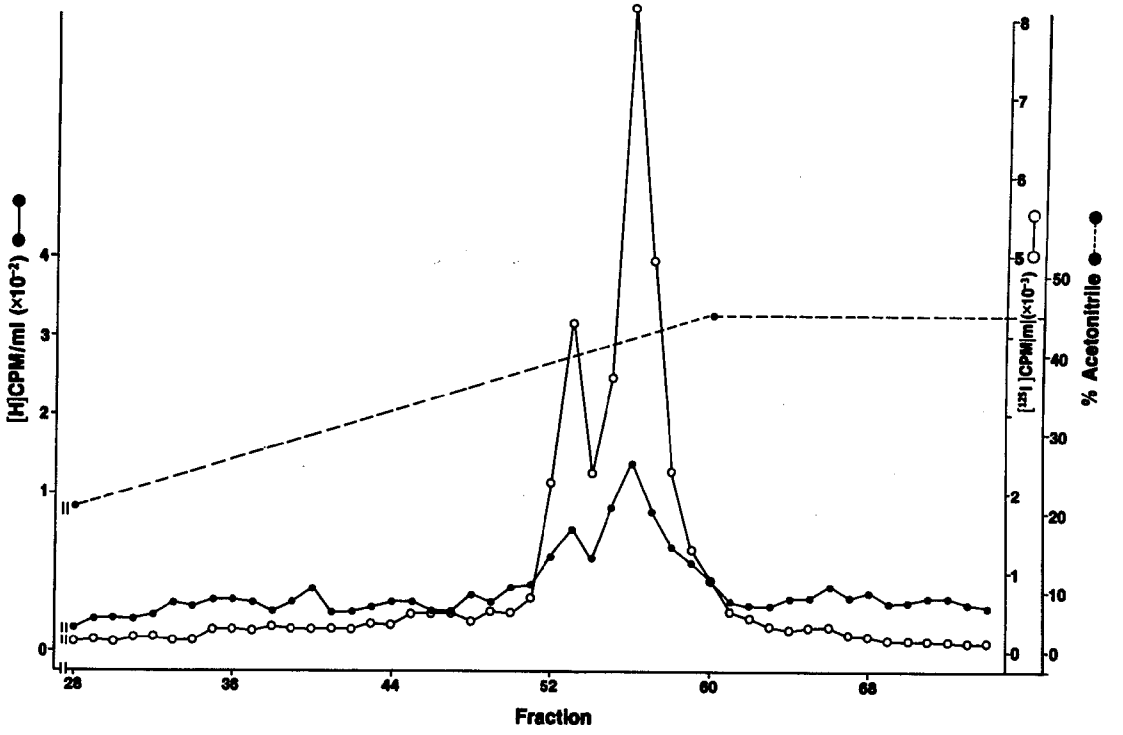

[H]CPM/ml (×10⁻²)
[125I]CPM/ml (×10⁻¹)
% Acetonitrile
Fraction

TABLE 9

Incorporation of [^{3}H]-leucine into total protein and anti-DGP-γ_3 affinity-bound protein

	-FCS			+FCS		
day	total soluble protein[1]	bound protein[2]	%[3]	total soluble protein	bound protein	%
2	1781.76	10.38	0.58	1516.16	9.34	0.62
4	1145.20	5.64	0.49	1133.20	5.29	0.47
6	4118.64	23.28	0.57	2205.52	17.99	0.82
8	4406.08	40.89	0.93	3772.32	25.68	0.68
10	2278.16	24.21	1.06	4134.80	33.58	0.81

[1] [^{3}H]-CPM/µg/8 molars

[2] [^{3}H]-CPM anti-DGP-γ_3-bound protein/µg/8 molars

[3] [^{3}H]-CPM bound protein/[^{3}H]-CPM total protein) x 100

FINAL DISCUSSION AND SUMMARY

Gla-containing proteins are abundant in bone and dentin. BGP and DGPs are highly homologous in terms of structure and presumably in terms of chemistry and mechanism of biosynthesis. It seems likely that functions would also be similar, although such a notion does not necessarily follow. An actual biological function for these Gla-containing proteins, however, has yet to be determined.

With any scientific question, the first problem to be faced is the development of a suitable model to allow for the appropriate research. It was this need that yielded the first objective of the present study. The major goal of this research was to gain further information regarding the possible physiological significance of the DGPs. Thus followed the need to develop an <u>in vitro</u> model of dentinogenesis and mineralization. The maxillary third molar from the young rat was used for this purpose. Molar germs were removed prior to (or perhaps coinciding with) terminal differentiation of odontoblasts and first elaboration of predentin matrix. At this stage no mineralized tissue was present within the molar. Molars were cultured in two conditions, in medium with or without supplementation with fetal calf serum. Appearance of a mineralized phase was evaluated biochemically and histologically. Molars cultured with serum developed and synthesized dentin and enamel matrices. These matrices mineralized to form dentin and enamel. Molars cultured without serum also continued to develop, but no mineral deposition occurred in these molars.

A model was thus available for study of DGPs in relation to the formation of mineral. Since Gla-proteins may be intimately involved in mineral homeostasis, it was of interest to examine DGPs relative to the onset of mineralization within the developing molar. DGPs were isolated by affinity chromatography or by gel filtration HPLC. DGPs were identified by reversed-phase HPLC and by SDS-PAGE. DGPs were identified from molars cultured both with and without serum. DGP biosynthesis, and total protein synthesis, generally increased in rate over the experimental period examined. DGPs represented approximately 0.5-1.0% of all acid- and guanidine/Tris-soluble proteins synthesized by molars. Molars in both culture conditions synthesized qualitatively similar proteins.

The above data were extended to identify which cell type within the molar was responsible for the biosynthesis of DGPs. In addition, since DGPs have a strong affinity for mineral, it was also of interest to note any extracellular sites in which DGPs were present. This localization was accomplished by indirect immunofluorescence using a highly specific polyclonal antibody raised against a purified dentin Gla protein preparation. This antibody was shown by RIA not to cross-react with any other dentin matrix non-collagenous proteins. DGP antigens were localized immunohistochemically in both mineralized and non-mineralized molars in odontoblasts and no other cells. Pre-odontoblasts did not react. DGP antigens were localized extracellularly only within mineralized molars and only in mineralized dentin. Predentin did not react.

This study was designed to initiate an investigation of the possible relationship between DGPs and the onset of mineralization in developing teeth. Several questions can be asked as follows: Is the biosynthesis of DGPs a sufficient condition to allow for mineral formation

(i.e., will DGPs induce mineralization)? Conversely, is the presence of mineral a necessary condition for the expression of DGP biosynthesis? The observation of DGP biosynthesis in non-mineralized molars clearly indicated that mineral crystals are not necessary for DGP biosynthesis. In addition, this same finding shows that the expression of DGP is not sufficient to induce mineralization in molars. The present study cannot, however, address the question as to whether DGPs are necessary for matrix mineralization. Such an investigation would require the incubation of molars in the presence of an inhibitor of DGPs (e.g., warfarin). A positive result (the formation of mineral) would show conclusively that mineralization can occur in the absence of γ-carboxylated DGPs. On the other hand, a negative result (no mineral formation) would be inconclusive. One could not be certain whether the lack of mineral was solely a result of the absence of DGPs or instead due to direct cytotoxic effects of the inhibitor *in vitro*.

Rat dental cells have been shown to synthesize DGPs (285). The immunohistochemical data presented in this study have shown that molar odontoblasts, and no other cell types, are responsible for DGP biosynthesis *in vitro*. A similar finding has been reported by Bronckers, *et al*. (174) using *in vivo* rat molar material. Of paramount interest is the mode of secretion of DGPs by odontoblasts and their possible extracellular functions. Are DGPs transported through the odontoblastic process and deposited directly at the mineralization front, as has been suggested for phosphoproteins (4,250) and glycoproteins (5)? This idea is supported by the present findings of immunostaining in dentin, but not in predentin, in agreement with Bronckers, *et al*. (174). However, such a transport process would imply that DGP antigenicity should also

be localized to odontoblastic processes. The present study has not shown any evidence for this staining, although odontoblastic process immunostaining for DGPs has been suggested (174). Such staining would require resolution at the electron microscopic level, a resolution beyond the scope of this research.

An alternate hypothesis is that DGPs are released from odontoblasts and bind to dentin because of their Gla-dependent affinity for HA (57-59), in a manner similar to the suggested binding of BGP in bone (76). Immunoreactive BGP can be localized in mineralized bone, but not in osteoid (74). The data reported here and by Bronckers, et al. (174) are consistent with this mode of secretion as well. Further experiments with mineralizing and non-mineralizing molars should give insight into this problem.

This study has presented a culture system in which explanted molar organs continued to develop and differentiate. Molars incubated in serum-supplemented medium mineralized de novo in culture. The biosynthesis of DGPs was demonstrated biochemically and immunohistochemically in both mineralized and non-mineralized molars, and terminally differentiated odontoblasts were the specific site for this biosynthesis. Finally, this study has raised further questions regarding DGPs and has developed a protocol useful for investigations of a number of dentin matrix components.

CONCLUSIONS

1. 11-Day rat maxillary third molars show *de novo* mineralization *in vitro* when incubated in the presence of FCS; molars cultured without FCS continue to develop but do not mineralize.

2. Molars cultured in serum-supplemented or serum-free medium show a general trend of increasing protein biosynthesis over a 10-day culture period and synthesize qualitatively similar proteins. There are quantitative differences in the biosynthesis of several proteins by molars incubated in mineralizing or non-mineralizing conditions.

3. Molars synthesize DGPs with or without mineralized dentin being present.

4. A rabbit anti-rat polyclonal antibody raised against a highly purified dentin Gla protein preparation, DGP-γ_3, is specific for DGPs and does not cross-react with any other dentin non-collagenous proteins.

5. Odontoblasts are the specific cell type responsible for DGP biosynthesis within the developing molar. Odontoblasts synthesize DGPs approximately coincidentally with the elaboration of predentin matrix and prior to the appearance of mineral.

6. In the molar organ, extracellular DGPs are found in mineralized dentin but not in predentin or at any other site.

SUGGESTIONS FOR FUTURE RESEARCH

The present study has identified several areas for further investigations. Further questions have been raised regarding the synthesis and secretion of DGPs. Are DGPs synthesized by odontoblasts in quantitatively similar amounts in both mineralized and non-mineralized molars? Are DGPs released intact into the media from non-mineralized molars or are they degraded? Is the method of secretion of DGPs by odontoblasts dependent on the presence of dentin; what precisely is the mechanism of secretion? Is the biosynthesis of DGPs stimulated by $1,25(OH)_2D_3$ as is the case for BGP?

The culture system described in this report presents a unique system for such investigations. The use of a defined medium allows for the addition of various metabolites [e.g., $1,25(OH)_2D_3$] or inhibitors (e.g., warfarin) in a precise and controlled manner. Alterations in the expression of molar development could then more accurately be ascribed to the perturbating agent. As suggested earlier, the mode of secretion of DGPs by odontoblasts is of major importance. The identification of DGPs in both mineralizing and non-mineralizing molars immediately points to research methods to address this problem. The present data show that mineralization is not necessary for the expression of DGP biosynthesis. What is the extracellular fate of DGPs in non-mineralized molars? Indeed, is mineral necessary for DGPs to be secreted at all, or are they instead degraded intracellularly? The answers to these questions would

give further insight into a possible role for this interesting group of proteins.

One of the major contributions of this research has been the establishment of an *in vitro* model of dentinogenesis and mineralization. Almost all of the developmental studies performed and proposed for DGPs are appropriate for other dentin matrix components as well. Since other dentin NCPs have been at least partially characterized, and biochemical methods exist for their identification, the above methods and questions can be applied to these NCPs as well. Such studies should provide much information regarding metabolism and functions for calcifying extracellular matrices.

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