Identification of Four Extracellular-Matrix Enamel Proteins during Embryonic-Rabbit Tooth-Organ Development

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1. Investigations were designed to identify the proteins which characterize the ameloblast phenotype, and to determine to what extent these extracellular-matrix proteins were degraded as a function of enamel matrix mineralization and maturation. 2. The identification of enamel proteins was based on comparisons between the electrophoretic patterns of enamel-containing and non-enamel-containing matrix extracts isolated from specific regions within 26-day embryonic New Zealand White rabbit incisor and molar tooth organs. 3. Since enamel proteins become mineralized on secretion, matrix specimens were demineralized in cold 5% (w/v) trichloroacetic acid, extracted with buffered 6M-urea and reduced with mercaptoethanol, and then the solubilized proteins were fractionated by urea/polyacrylamide-gel electrophoresis. 4. Three enamel-specific electrophoretic components were identified in newly secreted enamel-matrix specimens and this number increased as a function of mineralization and maturation. 5. Antibodies were prepared against embryonic rabbit extracellular matrix containing enamel. Comparison between immunoelectrophoretic patterns demonstrated that two of the three enamel components were antigenic. 6. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was used to identify four enamel proteins of mol.wts. (1) 65000 (2) 58000 (3) 22000 and (4) 20000, localized within enamel matrix. Enamel proteins (1) and (3) were phosphorylated, whereas (2) and (4) did not contain detectable phosphate. Labelled proline, leucine, tryptophan and glucosamine were incorporated into each of the four enamel proteins extracted from tooth explants incubated in the presence of radioactive precursors for 6 h. Whereas four proteins were identified in newly secreted enamel matrix, the concentrations of high-molecular-weight proteins (1) and (2) were found to decrease and the number (>10) and concentration of low-molecular-weight polypeptides increased as a function of advanced enamel-matrix mineralization and maturation.

An impressive number of biochemical studies have reported the presence of a large number of heterogeneous polypeptides called enamel proteins, enamelin or amelogenins in developing enamel matrix (Eggert *et al.*, 1973; Elwood & Apostolopoulos, 1975*a,b,c*; Fincham, 1971; Fukae & Shimizu, 1974; Katz *et al.*, 1965; Mechanic, 1971; Nikiforuk & Gruca, 1971; Weidmann & Eyre, 1971). Bovine and pig foetal enamel proteins, for example, have been reported to range in mol.wt. from 25000 to 3000 and to consist of 9–22 different electrophoretic components. Enamel proteins are described as being glycosylated (approx. 1% glucosamine and galactosamine) and phosphorylated (Seyer, 1972; Seyer & Glimcher, 1971). These proteins have similar and distinctive amino acid compositions (e.g. 25% proline, 17%

glutamic acid, 9% leucine, 7% histidine, 6% methionine and trace amounts of tryptophan), and are devoid of either hydroxyproline or hydroxylysine (Eastoe, 1960). A severe limitation of previous studies, however, was that the foetal enamel matrix proteins which were analysed were obtained from mineralized enamel matrix specimens with various extents of enamel maturation. Therefore the specimens used in such studies probably contained a large number of heterogeneous polypeptides of different molecular weights resulting from the degradative processes associated with enamel mineralization and maturation.

The basic problem in which our laboratory has been interested is how many enamel-protein gene products characterize the ameloblast phenotype, and to what extent and in what manner are these proteins degraded as a function of mineralization and maturation? Our experimental strategy was to design

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investigations which would use the developmental pattern present within the embryonic tooth organ. The selection of a method by which to identify embryonic enamel matrix proteins is by no means easy. Secreted proteins are quickly mineralized and subsequently degraded during enamel maturation. Perhaps the only means of circumventing such difficulties is to isolate the newly formed extracellular matrices, and to divide them into various segments containing a spectrum ranging from primitive matrix, which does not contain enamel, to segments that contain relatively mature enamel. Comparison of the protein patterns for each matrix segment could then provide a method of identifying enamel proteins and distinguishing these from proteins extracted from the adjacent pre-dentine and dentine. Antisera against embryonic-rabbit enamel matrix were used to facilitate further the identification of enamel proteins.

Experimental

Materials

Eagle's Minimum Essential Medium (Eagle, 1959), foetal calf serum and Dulbecco's phosphate-buffered saline (Dulbecco & Vogt, 1954) were all purchased from Grand Island Biological Co., Grand Island, NY, U.S.A. Urea (ultra pure), Tris base (99.5% pure), 2-mercaptoethanol, dithiothreitol and boric acid were purchased from Schwarz-Mann, Orangeburg, NY, U.S.A. All supplies required for polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS), and the AG 501-(X8) resin were supplied by Bio-Rad, Richmond, CA, U.S.A. Radioactive compounds were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Their specific radioactivities were as follows: L-[U- ^{14}C]proline, 260 mCi/mmol; L-[4,5- ^3H]leucine, 500 mCi/mmol; L-[2,3- ^3H]tryptophan, 200 mCi/mmol; D-[1- ^{14}C]glucosamine, 5–10 mCi/mmol; and Na ^{125}I , carrier-free. Xylene and Triton X-100 used in liquid-scintillation counting fluids were purchased from Beckman Instrument Co., Fullerton, CA, U.S.A. Omnifluor was supplied by Research Products International, Elk Grove Village, IL, U.S.A. All other chemicals and reagents were of the highest available purity, and glass-distilled water was used in the preparation of all solutions.

Preparation of enamel-containing and non-enamel-containing extracellular-matrix

(a) *Age determination of the embryonic tooth organs.* Tooth organs were obtained as a routine from New Zealand White rabbit embryos after 26 days gestation. The age of the embryos was provided by the supplier (ABC Caviary, Pomona, CA, U.S.A.).

(b) *Dissection of incisor and molar tooth organs.* Pregnant rabbits were killed by injecting 20 ml of air into the large vein on the mesial margin of either ear. The embryos were removed by laparotomy and immediately covered with ice. In a typical experiment eight to ten embryos were removed from each of three pregnant rabbit does. Mandibular and maxillary incisor and mandibular first and second molar tooth organs were removed with the aid of a stereoscopic dissecting microscope and washed with five 1 ml portions of cold Dulbecco's phosphate-buffered saline. Molar tooth organs intended for tissue culture and radioactive-labelling experiments were transferred at this time into Eagle's Minimum Essential Medium, pH 7.4, and stored at 4°C for no longer than 1 h before initiating labelling experiments.

(c) *Microdissection of incisor tooth-organ extracellular matrices.* After removal of the dental papillae, incisors were placed with their labial matrix surfaces oriented down in plastic dishes containing cold Dulbecco's phosphate-buffered saline, pH 7.4. Four sequential cuts were then made with a micro-scapel. First, the tip segment of the matrix was cut free by transecting the matrix perpendicular to the longitudinal axis, approx. 250 μm below the apex. Next, the cervical matrix segment was removed by a cut 800 μm above the basal end of the incisor (Fig. 1).

Fig. 1. Diagram of the scheme used to dissect 26-day embryonic rabbit incisor extracellular matrices into incisal tip, labial, lingual and cervical segments

Each matrix segment represented a distinct morphological, developmental and biochemical zone. The cervical matrix did not contain enamel, whereas the labial matrix segment contained newly synthesized and secreted enamel proteins. The incisal tip matrix segment contained a more mature enamel protein population.

Scheme 1. Flow diagram of experimental procedures for extraction and identification of newly secreted embryonic enamel proteins

Finally, two cuts were made slightly oblique to the longitudinal axis along the edges of the remaining transitional segment of the extracellular matrix. The outer edges of these segments were discarded.

(d) *Sonication of the isolated extracellular matrices.* The physical removal of the dental papillae resulted in extracellular matrices with various amounts of adherent cells. Cell contamination was subsequently removed by using low-power sonication as previously reported by our laboratory (Guenther *et al.*, 1975; Slavkin *et al.*, 1970). The sonicated matrix segments were thoroughly washed in Dulbecco's phosphate-buffered saline and freeze-dried. Portions of the dried matrix segments were weighed on a Cahn RG Electrobalance (Cahn Instruments, Cerritos, CA, U.S.A.) before starting the extraction scheme (Scheme 1).

(e) *Trichloroacetic acid demineralization of sonicated matrices.* Preweighed samples of matrix segments were transferred into polystyrene tubes (13 mm × 75 mm) containing cold 5% (w/v) trichloroacetic acid; 2 ml of solution was used for 20–100 matrices. The tubes were shaken at 60 oscillations/min at 4°C for 40 min. The supernatant was subsequently dialysed against cold 0.5% acetic acid and assayed for protein content (Hartree, 1972; Lowry *et al.*, 1954). The demineralized matrix segments were then washed with two 2 ml portions of methanol to remove residual trichloroacetic acid. The freeze-dried matrix segments were reweighed as a routine to determine the percentage of mineral loss attributed to the demineralization procedure (Fig. 2).

Fig. 2. Evaluation of the trichloroacetic acid-demineralization procedure

Incisal tip segments were selected to determine the shortest time in which demineralization was completed after the addition of cold 5% trichloroacetic acid. After 30 min the weight loss reached a plateau, indicating maximum weight loss owing to demineralization. No protein was detected in the trichloroacetic acid supernatants.

(f) *Extraction of tooth extracellular-matrix proteins.* Demineralized matrix segments were extracted with 6M-urea in 100mM-Tris/borate buffer, pH 8.6 (7 µg of demineralized matrix/µl of Tris/borate-buffered urea solution). Extraction was carried out at 60°C for 90 min. The solubilized proteins were fractionated electrophoretically within 2–4 h after recovery (Guenther *et al.*, 1975). The matrix residue resulting from the urea extraction was weighed as a routine. The yield of solubilized matrix proteins was determined by subtracting the mass of the matrix residue, resulting from the urea extraction, from the mass of the demineralized matrix segments.

(g) *Scanning-electron-microscopy criteria.* After each preparative step, matrix samples were individually fixed by immersion in formaldehyde/glutaraldehyde buffered with 0.2M-sodium cacodylate, pH 7.2, for 1 h at 4°C. After fixation, the matrix segments were processed for critical-point drying (Turner & Green, 1973), and then a gold surface coating was applied with an Edwards high-vacuum apparatus. The specimens were then examined and photographed in a Cambridge Stereoscan electron microscope, model S4-10.

Electrophoretic analysis of enamel proteins

(a) *Polyacrylamide-gel electrophoresis.* Dissolved proteins were fractionated by polyacrylamide-gel electrophoresis in the presence of urea or SDS. In both gel systems the final acrylamide concentration was 7.5% (w/v) and the dimethylenebisacrylamide concentration was 0.2%. Gel stock solutions were: (A) 50% (w/v) acrylamide; (B) 2% (w/v) dimethylenebisacrylamide; (C) Tris/borate buffer, pH 8.6 (64.0 g of Tris base and 29.2 g of boric acid per 500 ml, adjusted to pH 8.6 if necessary); (D) 10M-urea; (E) 10% (w/v) ammonium persulphate (prepared fresh daily); (F) NNN'-tetramethylethylenediamine.

To prepare 10 ml of 6M-urea/polyacrylamide-gel solution the stock solutions were mixed in the following order and volumes: (A) 1.5 ml; (B) 1.0 ml; (C) 1 ml; (D) 6 ml; (E) 0.02 ml; (F) 0.0015 ml; deionized water was used to adjust the volume to 10 ml. To prepare 0.1% SDS/polyacrylamide-gel solutions, the procedure was identical except that 0.1 ml of 10% (w/v) SDS was used and the urea concentration was decreased to a final concentration of 1 M. The electrode buffers used for gels containing urea and SDS were respectively 100mM-Tris/borate buffer and 100mM-Tris/borate buffer plus 0.1% (w/v) SDS. Gels were pre-run as a routine at a constant voltage of 200 V for 1 h.

All samples were adjusted to 400mM-2-mercaptoethanol or 400mM-dithiothreitol and heated at 60°C for 30 min in an atmosphere of 100% N₂ before electrophoresis. For electrophoresis, 60 µg of protein

was applied to each gel. The urea/polyacrylamide gels were electrophoresed for 5 h, whereas the SDS/polyacrylamide gels were run only until the tracking dye, Bromophenol Blue, reached the bottom of the gel. Gels were fixed and stained in a solution of 0.25% (w/v) Coomassie Brilliant Blue R-250, 9.2% (v/v) acetic acid and 40% (v/v) methanol for 1 h, and destained with a solution of 7.5% (w/v) acetic acid and 25% (v/v) methanol. The distribution of radioactivity in gels was determined by slicing the gels into 1.0 ± 0.1 mm slices, placing slices into vials and then treating with 0.5 ml of 3% (w/v) H_2O_2 solution for 2 h at 60°C before adding 3.0 ml of scintillation fluid. The scintillation fluid consisted of 3.0 g of Omnifluor/litre of xylene/Triton X-100 (3:1, v/v). Samples were counted for radioactivity with a Beckman LS-230 liquid-scintillation spectrometer.

(b) *SDS/polyacrylamide-gel molecular-weight markers.* Markers for the determination of enamel-protein molecular weights by SDS/polyacrylamide-gel electrophoresis were obtained by cross-linking myoglobin with glutaraldehyde (Payne, 1973), resulting in a heterogeneous mixture of monomers, dimers, trimers, tetramers and pentamers (mol.wts. 16900, 33800, 50400, 67600 and 84500 respectively). In replicate experiments, embryonic-rabbit skin collagen α chains were used as molecular-weight markers on SDS/polyacrylamide-gel electrophoresis (Trelstad & Slavkin, 1974; Slavkin *et al.*, 1975).

Biosynthesis and secretion of embryonic enamel proteins in vitro

In experiments designed to study protein biosynthesis and secretion into the forming enamel matrix, groups of 20–30 molar tooth organs were incubated for 6 h at 37°C in 1.0 ml of serum-free Eagle's Minimum Essential Medium containing one of the following radioactive precursors: L-[U- ^{14}C]proline (10 $\mu\text{Ci/ml}$); L-[4,5- ^3H]leucine (50 $\mu\text{Ci/ml}$); L-[2,3- ^3H]tryptophan (50 $\mu\text{Ci/ml}$); or D-[1- ^{14}C]glucosamine (10 $\mu\text{Ci/ml}$). After incubation, labelled tooth organs were washed with five 1 ml portions of Dulbecco's phosphate-buffered saline. Extracellular matrices were then isolated, sonicated and demineralized.

The trichloroacetic acid supernatant was fractionated by Sephadex G-15 chromatography. A column (1 cm $\times$ 10 cm) was equilibrated with 0.5 M-acetic acid containing 0.3% sodium dodecyl sulphate. The void-volume fraction was examined for molecules containing [^{14}C]proline, [^{14}C]glucosamine, [^3H]leucine or [^3H]tryptophan, concentrated and either subjected to SDS/polyacrylamide-gel electrophoresis or analysed for protein content. Subsequently the labelled molar extracellular matrices were extracted with urea and then fractionated electrophoretically as described above.

Chemical determination of phosphorylated extracellular-matrix proteins

The object of this method is to measure organically bound phosphate after it has become converted into P_i . The P_i then reacts with ammonium molybdate, forming phosphomolybdate, which after reduction with ascorbic acid gives a blue-coloured complex (Keleti & Lederer, 1974). Urea-soluble proteins in rabbit incisor-tooth extracellular matrices were fractionated by polyacrylamide-gel electrophoresis in the presence of urea or SDS, as described above. After the gels were fixed and subsequently sliced in 1 mm discs, the individual gel discs were mixed with 100 μl of a $\text{HClO}_4/\text{H}_2\text{SO}_4$ solution [6.5 ml of 70% (v/v) HClO_4 , 50 ml of 10 M- H_2SO_4 , adjusted to 100 ml of deionized water] and warmed first to 95°C for 2 h, followed by an additional 2 h at 165°C. NaH_2PO_4 (1.2 g/litre) was used as a standard. After the samples were cooled to room temperature, 1 ml of buffered ammonium molybdate solution was added [0.1 M-sodium acetate, 25% (w/v) ammonium molybdate, 1% ascorbic acid] and, after 2 h of incubation at 37°C, the A_{280} of samples was read (Keleti & Lederer, 1974).

Preparation of rabbit alloantiserum against embryonic enamel-containing extracellular matrix

Alloimmune serum was prepared in young female New Zealand White rabbits against molars and incisors of embryonic New Zealand White rabbits at 26 days of gestation (Schonfeld, 1975). The matrices were sonicated as described above, washed with Dulbecco's phosphate-buffered saline, rinsed with deionized water, and then frozen in liquid N_2 and pulverized in a liquid-nitrogen mill. Powdered matrix was suspended in complete Freund's adjuvant at a concentration of 2 mg of matrix material per ml of adjuvant. Rabbits were immunized by giving weekly injections for 6 weeks. Control serum was prepared by administering complete Freund's adjuvant by the same procedures used for the matrix adjuvant suspension. The anti-(matrix γ -globulin) was prepared from the serum by $(\text{NH}_4)_2\text{SO}_4$ precipitation (Campbell *et al.*, 1970).

Gel-electrophoresis-radioimmunoassay of enamel proteins

Urea/polyacrylamide-gel electrophoresis was performed on extracts of matrices as described above, except that, whereas the analytical gels were loaded with 60 μg of protein, gels for immunoelectrophoresis were loaded with 160 μg of protein. A few representative gels were fixed and stained, whereas most of the gels were placed into 100 mM-phosphate buffer, pH 7.0, for 36 h at room temperature. The buffer was changed during this incubation to allow urea to

diffuse out of the gels. Since enamel proteins have been characterized as being insoluble in neutral salt solutions of low ionic strength, it is unlikely that enamel proteins would have been lost during the incubation conditions. As a routine, representative gels were fixed and stained after incubation in phosphate buffer, indicating that no protein bands were lost. After incubation in phosphate buffer, gels were then incubated for 72h at room temperature with either anti-molar or -control γ -globulins which had been iodinated with Na^{125}I (carrier-free) (McConahey & Dixon, 1966). The specific radioactivities of the γ -globulins were: anti-matrix, 430000 c.p.m./mg of protein; control, 100000 c.p.m./mg of protein. Antibody that has not reacted was allowed to diffuse from the gels by placing them into 100mm-phosphate buffer, pH 7.6, for 96h. The buffer was changed during this incubation to facilitate the diffusion of antibodies that had not reacted. The gels were then sliced and counted for radioactivity in a liquid-scintillation spectrometer.

Results

Isolation of enamel-matrix proteins

In previous studies, trichloroacetic acid was reported to be effective for demineralizing tooth extracellular matrices without solubilizing proteins (Guenther *et al.*, 1975). In the present study we noted that the trichloroacetic acid supernatant did not contain detectable protein or any of the radioactive precursors used in the biosynthesis studies *in vitro*. In addition, no protein was detected in the methanol wash.

Tris/borate-buffered 6M-urea extracts from enamel-containing matrices contain three major electrophoretic components, as shown by urea/polyacrylamide-gel electrophoresis (Figs. 3 and 4). Scanning-electron-microscopic observations of the surfaces of enamel-containing matrices indicated the complete removal of enamel after extraction with buffered 6M-urea (Guenther *et al.*, 1975). Urea extraction

methods removed 40% of the enamel-containing labial-matrix-segment proteins and 41% of the lingual-matrix-segment proteins (Table 1); essentially all non-collagenous proteins were extracted. In each matrix segment the insoluble residue remaining after urea extraction contained essentially 100% insoluble collagen. Further, we recovered 95% of the total radioactivity incorporated into the extracellular matrices after incubation of molar explants with various radioactive precursors for 6h.

Identification of newly secreted enamel-matrix proteins

Urea/polyacrylamide-gel electrophoresis. Enamel-matrix proteins were identified by comparing enamel-containing with non-enamel-containing urea extracts by urea/polyacrylamide-gel electrophoresis. Analysis of gel patterns from each of the matrix segments demonstrated that most of the constituents shown by electrophoresis were common to all segments. The cervical matrix segment contained progenitor dentine matrix constituents but did not contain enamel matrix. Three major enamel-matrix constituents were identified because they were present in enamel-containing segments but were not detectable in non-enamel-containing matrix segments (Fig. 3). The incisal tip segment contained increasing amounts of lower-molecular-weight constituents, indicative of the enamel-matrix maturation process.

Gel-electrophoresis-radioimmunoassay. Alloimmune antiserum was prepared against embryonic-rabbit enamel proteins by methods developed in our laboratory (Schonfeld, 1975). Indirect immunofluorescence microscopy has indicated that the alloantisera contained antibodies specific for the enamel matrix (Schonfeld, 1975).

For gel-electrophoresis-radioimmunoassay, 160 μg of total urea-extracted protein was loaded for urea/polyacrylamide-gel electrophoresis. After electrophoresis the gels were incubated in 100mm-phosphate buffer for 36h to remove urea as described above. No protein bands were lost as a result of this procedure. After the incubation with phosphate buffer, either

Fig. 3. Analysis of enamel protein by urea polyacrylamide-gel electrophoresis

The electrophoretic patterns represent proteins found in urea extracts of labial (la) and cervical (ce) matrix segments. The arrows indicate bands representing three proteins found in extracts of the labial (enamel-containing) but not in the cervical (enamel-devoid) matrix segments. These three protein bands (representing enamel proteins) were also seen on electrophoresis of extracts from the lingual segment, but the proteins were present in a much lower concentration.

Fig. 4. Molecular-weight determinations for embryonic enamel proteins

Urea extracts of various tooth matrix segments were electrophoresed on SDS/polyacrylamide-gels after treatment of the extracted proteins with SDS. The gels represent: (a) lingual matrix segment, (b) cervical matrix segment, (c) labial matrix segment and (d) incisal tip segment. (e) For molecular-weight markers, myoglobin chains were cross-linked with glutaraldehyde to produce a mixture of monomers (16900), dimers (33800), trimers (50400), tetramers (67600) and pentamers (84500). Note that four enamel proteins (1, 2, 3 and 4) were resolved in this electrophoretic system, as indicated in the gel of the enamel-containing labial matrix (c).

^{125}I -labelled anti-(enamel matrix) or anti-control γ -globulins were incubated with the various gels. The anti-(enamel matrix) antisera specifically reacted with two of the three major electrophoretic constituents characteristic of enamel proteins (Fig. 5). No antibody-antigen binding was detected in the urea/polyacrylamide-gel patterns treated with control serum (Fig. 5).

SDS/polyacrylamide-gel electrophoresis. This technique was used to fractionate further and identify the constituents of the urea-extracted protein mixture according to their molecular weights (Fig. 4). The gel patterns of the labial matrix segment identified two protein bands with mol.wts. of 65000 and 58000. Two additional protein bands (numbered 3 and 4 in Fig. 4) migrated with mol.wts. of 22000 and 20000. These

Table 1. *Mineral and protein content in developing regions of the extracellular matrices from 26-day embryonic incisor tooth organs*

To measure total mineral content, dry-weight determinations were made of washed, sonicated, cell-free matrices before and after two extractions with 5% trichloroacetic acid for 45 min at 4°C on a Cahn electrobalance. Percentages represent the averages of five determinations which were not different at a 95% confidence level. Each determination was made on segments obtained from 30 tooth organs. Protein content of the urea extract was determined from the dry weights of trichloroacetic acid-demineralized matrices before and after extraction with Tris/borate-buffered 6M-urea, pH 8.6. The urea extract contained essentially all of the non-collagenous proteins and collagen. Hydroxyproline determinations on the remaining residue after urea extraction indicated that the residue was entirely insoluble collagen (Slavkin *et al.*, 1967).

Extracellular matrix segment	Average length (μm)	Total mineral content (%)	Urea-extractable protein content		
			Collagen (%)	Non-collagenous proteins (%)	Collagenous/non-collagenous protein ratio
Tip	250 (± 5)	78.4	57.7	42.3	1.36:1
Labial	2000 (±15)	78.7	59.1	40.9	1.44:1
Lingual	1800 (±10)	78.6	59.9	40.1	1.49:1
Cervical	800 (± 7)	63.3	62.2	37.8	1.64:1

four proteins were not evident in the cervical matrix segment, and were only present in trace amounts in the lingual matrix segment. The labial and tip portions both contained the enamel protein of mol.wt. 58000. Enamel protein (numbered 1 in Fig. 4c) was evident only on the SDS/polyacrylamide gels of the labial matrix segment, indicating the largest newly secreted enamel protein; this segment represents the region of overt enamel-protein synthesis and secretion in the developing incisor tooth. The incisal tip segment represents older enamel matrix and was used to indicate enamel maturation.

Phosphorylated enamel proteins. Phosphate analysis demonstrated that only the 65000- and 22000-mol.wt. proteins contained covalently bound phosphate (Fig. 6). The other two enamel proteins were not phosphorylated. Phosphate was also associated with dentine collagen as previously reported (Veis, 1972), and was also observed in association with several other proteins (e.g. alkaline phosphatase and dentine phosphoproteins).

Biosynthesis and secretion of enamel-matrix proteins *in vitro*. Radioautographic studies (Belanger, 1956; Cotton & Hefferren, 1966; Frank, 1970; Greulich & Slavkin, 1965; Hwang *et al.*, 1961; Slavkin *et al.*, 1968, 1976; Warshawsky, 1966; Weinstock & LeBlond, 1971; Weinstock & Young, 1972) have shown that exogeneous amino acids, carbohydrates and SO_4^{2-} are incorporated into newly secreted enamel organic matrix *in vivo* in a number of different laboratory animals. The kinetics of various radioactive precursors (e.g. fucose, glucosamine, proline, tryptophan) during the secretory phases of amelogenesis have been studied with electron-microscopic

radioautography; translation of enamel proteins, post-translational modifications, packaging into secretory vesicles, intracellular transport and secretion to form the enamel matrix requires approx. 30 min (see reviews by Weinstock, 1972; Slavkin, 1974). The present studies were designed to evaluate whether the labelled macromolecules secreted by ameloblast cells *in vitro* were enamel proteins by the criteria described above.

Embryonic molar explants were incubated in the presence of radioactive precursors for 6 h. The relative metabolic activity of the secretory ameloblasts was determined by measuring the incorporation of radioactivity in buffered urea extracts of matrices. The mixtures of labelled macromolecules in the extracts were further separated by polyacrylamide-gel electrophoresis in the presence of urea or SDS, and the gel patterns of labelled molecules were compared with patterns of unlabelled molecules that have been discussed above. The urea extracts of matrices from tooth organs, labelled with proline, leucine, tryptophan or glucosamine for 6 h *in vitro*, have two major radioactive components as detected on urea/polyacrylamide-gel electrophoresis. We have previously identified these components as enamel-matrix proteins (Fig. 3).

In contrast with the urea/polyacrylamide-gel system used in the present studies in which collagen does not enter the gels, essentially all of the urea-extracted proteins were fractionated by SDS/polyacrylamide-gel electrophoresis. The SDS/polyacrylamide-gel profiles of labelled enamel proteins were identical with those obtained by using unlabelled extracellular-enamel-matrix preparations. Each of

Fig. 5. Gel-electrophoresis-radioimmunoassay of urea extracts of incisor-tooth extracellular matrices

Approx. 160 μ g of protein was loaded on each gel and the proteins were electrophoresed as described. Urea was washed out of the gel, and the 125 I-labelled control serum (a) or anti-(enamel protein) serum (b) was allowed to diffuse into the gels. Antibodies that had not reacted were washed out, the gels were sliced into 1 mm discs and counted for radioactivity in a liquid-scintillation counter. The electrophoretogram represents urea/polyacrylamide-gel patterns, as in Fig. 3. Note that two of the three enamel proteins which can be resolved in this electrophoretic system bind the anti-(enamel protein) serum. Binding of control serum in these areas of the gel was not observed. Both of the iodinated sera were found to be associated to proteins located at the top of the gel. It is not clear whether this represents non-specific trapping of the sera in the band of precipitated protein found at the tops of the gels or whether this observation reflects the presence of naturally occurring alloantibodies to slowly migrating proteins.

the exogenous radioactive precursors was incorporated into all four enamel proteins during the 6h of organ culture (Fig. 7).

Discussion

The present investigation has identified four proteins within the enamel matrix of embryonic-

rabbit incisor and molar tooth organs. After demineralization of isolated matrices with trichloroacetic acid, enamel proteins were readily extracted with 6M-urea in Tris/borate buffer, pH 8.6, at 60°C. It was found essential to reduce urea extracts with either 400mM-mercaptoethanol or 400mM-dithiothreitol before electrophoresis on 7.5% polyacrylamide gels containing urea or SDS.

Fig. 6. Bar diagram representing the distribution of covalently bound phosphate associated with 6M-urea-extracted proteins from embryonic incisor teeth, fractionated according to molecular weights on 7.5% (w/v) polyacrylamide gels containing SDS

Phosphate was determined in individual 1 mm slices. It was found to be covalently bound to enamel proteins 1 and 3 of Fig. 4(c). Enamel proteins 2 and 4 of Fig. 4(c) did not contain detectable phosphate. The relative amount of phosphate is expressed in units of A_{820} per gel slice.

The approximate mol.wts. determined for the enamel proteins were (1) 65 000, (2) 58 000, (3) 22 000 and (4) 20 000. Enamel proteins (1) and (3) were both phosphorylated, whereas proteins (2) and (4) did not contain detectable phosphate. All four enamel-matrix proteins were synthesized and secreted by molar tooth organs *in vitro*, and each of these proteins incorporated radioactively labelled glucosamine in addition to radioactively labelled proline, leucine and tryptophan. Puromycin, in the culture medium at 2 mM, caused 95% inhibition of the incorporation of labelled amino acids and glucosamine into the enamel-matrix proteins over a 6 h incubation period. The inhibitory effects of puromycin on the incorporation of labelled precursors suggested that continuing protein synthesis was a prerequisite for the secretion of enamel-matrix glycoproteins.

Our investigations have provided results that complement the evidence reported by Fukae and his co-workers (Fukae, 1970; Fukae & Shimizu, 1974; Fukae *et al.*, 1972) and also offer an experimental

approach to explore the initiation and regulation of embryonic mammalian amelogenesis. Fukae (1970) had reported that young rats incorporated [^3H]-proline *in vivo* into one enamel protein within 30 min after the injection of the labelled amino acid. Shimokawa & Sasaki (1975) presented preliminary results which indicated that foetal ameloblast cells synthesized and secreted a relatively large enamel protein (mol.wt. >50 000), and subsequently a smaller protein was detected (mol.wt. 25 000), as determined by using SDS/polyacrylamide-gel electrophoresis. They suggested a possible precursor-product relationship as a post-translational modification of the enamel-protein gene product.

The degree of heterogeneity of proteins found within enamel extracellular matrix has been described in a number of conflicting reports. The discrepancies between our observations and those reported elsewhere may be due to the striking differences in the animal sources used to obtain the enamel matrix, the developmental stages of the enamel matrix selected, and the techniques used to identify the enamel-matrix proteins. In the vast majority of studies the separation of the enamel matrix from adjacent dentine was attempted by physical methods that invariably introduced cross-contamination and activated proteinases. A factor of even greater concern has been the continued use of foetal bovine and pig tooth organs in which the crown of the tooth and enamel formation (i.e. amelogenesis) had already been completed.

On secretion, enamel proteins are precipitated, and participate in the formation of calcium hydroxyapatite crystal formation and mineralization (Weinstock & LeBlond, 1971; Zipkin, 1970). Concomitant with increased mineralization is a degradation of enamel proteins (Fukae & Shimizu, 1974), a loss of protein and water, and an appreciable increase in the inorganic mass of the extracellular matrix.

The task of preparing embryonic enamel matrix and purifying soluble enamel proteins has proved to be extremely difficult and replete with technical difficulties (see the review edited by Fearnhead & Stack, 1971). Most of the modern methods for studying the biochemical properties of protein molecules were developed for soluble proteins. Enamel proteins are naturally insoluble under physiological conditions in that they are constituents of a thixotropic gel in association with forming calcium hydroxyapatite crystals (Eastoe, 1963). Very little if any protein can be extracted unless the enamel matrix is first demineralized. Like keratins (Fraser *et al.*, 1972), unless the enamel proteins are further modified during extraction, they will re-form into complex aggregates when the extraction medium is removed or diluted. These requirements are crucial if studies are designed to determine the actual number of enamel gene products which characterize the

Fig. 7. Incorporation of radioactive precursors into embryonic molar tooth organs *in vitro*

Embryonic tooth organs were cultured for 6 h with (a) glucosamine and leucine or (b) tryptophan and proline. The cultured teeth were demineralized and extracted with urea; urea extracts were subsequently electrophoresed on SDS-containing gels. The electrophoretic patterns were identical with those in Fig. 4. All of the radioactive precursors were incorporated into each of the four enamel-specific bands.

secretory-ameloblast-cell phenotype. To circumvent some of these technical difficulties it is imperative to demineralize the forming enamel matrix, to use

denaturing agents such as 6M-urea, and to further solubilize the proteins with reducing agents such as mercaptoethanol,

The immunological characteristics of enamel proteins are of some interest. Previously enamel protein was shown to be antigenic (Nikiforuk & Gruca, 1971). On secretion, enamel proteins become incorporated into a thixotropic gel in which mineralization is rapidly occurring. Newly secreted embryonic enamel proteins are essentially sequestered molecules within a mineralizing extracellular matrix and are not in contact with the host's immune system (Schonfeld, 1976). Our laboratory began a series of studies to investigate the possibility that enamel matrix might be immunogenic and capable of producing an alloantiserum against embryonic enamel matrix. Schonfeld (1975) produced an alloantibody directed against embryonic enamel matrix. Embryonic-rabbit tooth extracellular matrices were used as antigens in an immunization schedule in which the immunogen in Freund's adjuvant was injected into young female New Zealand White rabbits over a 6-week period. This procedure resulted in a high titre of antibody which was found by indirect immunofluorescence microscopy to be specific for newly secreted enamel matrix (Schonfeld, 1975). Subsequent studies using gel electrophoresis-radioimmunoassay demonstrated that, of the three major constituents observed on urea/7.5% polyacrylamide-gel electrophoresis, characteristic for embryonic enamel matrix, two of these constituents were antigenic and reacted with the anti-(enamel matrix) antisera (Fig. 5).

The available data indicates that one major gene product characterizes the extracellular-matrix phenotype associated with embryonic ameloblast cell differentiation. This protein has appreciable post-translational modifications during the course of biosynthesis, secretion, extracellular-matrix formation and mineralization (see reviews by Weinstock, 1972; Slavkin, 1974). It is also suggested that the heterogeneity reported among the polypeptides in mature and older enamel reflect proteinase degradation associated with enamel maturation. We interpret our observations to indicate that embryonic amelogenesis is chemically characterized by a precursor enamel protein (Slavkin *et al.*, 1976) which is proteolytically degraded into a large number of increasingly smaller polypeptides (M. Fukae, personal communication) concomitant with enamel maturation. We predict that the phosphorylated 65000-mol.wt. enamel protein is the precursor of the 58000-, 22000- and 20000-mol.wt. polypeptides.

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