Articles

Phosphoproteins of Bovine Dentin: Evidence for Polydispersity during Tooth Maturation†

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ABSTRACT: The ethylenediaminetetraacetic acid extractable phosphoproteins of dentin from very young fetal calves consisted principally of a monodisperse species with an apparent molecular weight of at least 94 000 and a phosphorus content of 8.0% (w/w). In contrast, the soluble phosphoproteins extracted from mature adult animals under indentical conditions ranged in molecular weight from 18 000 to 94 000 and contained less phosphorus. The data support the conclusion that the phosphoproteins of bovine dentin are degraded in vivo during mineralization and maturation of the tissue.

Polyanionic phosphoproteins having an abundance of both aspartyl and phosphoseryl residues are the major noncollagenous proteins of the extracellular matrix of dentin (Veis & Perry, 1967; Veis et al., 1972; Linde et al., 1980). Molecular weight estimations for isolated phosphoproteins have varied considerably in different laboratories (Veis et al., 1972; Lee et al., 1977; Jontell & Linde, 1977; Dimuzio & Veis, 1978; Termine et al., 1980). It has been recently suggested that the wide range in the reported molecular weight estimations may be a consequence of protein degradation during the progressive increase in mineralization of dentin which accompanies tooth maturation (Veis et al., 1981), similar to the degradation of the phosphoproteins of tooth enamel (Strawich & Glimcher, 1982). To investigate the possible in vivo proteolysis of the phosphoproteins during tooth maturation, we have compared the molecular weight distribution of the ethylenediaminetetraacetic acid (EDTA)[1] extractable phosphoproteins obtained from two widely separated stages in the development of bovine molar teeth—teeth from fetal calves and from adult steers.

Materials and Methods

General. All preparative procedures, including Sephadex G-100 and DEAE-cellulose chromatography, but excluding pH measurements, were conducted at 4 °C. Measurements of pH were made at ambient temperatures. To minimize bacterial growth, freshly prepared buffers were sterilized by autoclaving, and chromatographic columns were washed with 70% ethanol prior to packing.

Preparation and Desalting of Dentin EDTA Extracts. Unerupted molars from 3- to 4-month-old fetal calves and erupted molars from 2-year-old steers were dissected free of adherent soft tissues and stirred briefly in phosphate-buffered saline, pH 7.4, (30 mM Tes, 150 mM NaCl) with 0.1% sodium azide. To remove the outer enamel layer, teeth were scraped with a scalpel, ground with a dental burr, and then soaked overnight in 0.05 M NH_4HCO_3, pH 7.9. After being lyophilized, the dentin was pulverized in a liquid N_2 cooled Spex mill and the resultant powder demineralized by stirring in 5 volumes of 0.5 M EDTA and 0.05 M Hepes, pH 7.5, with the following proteinase inhibitors: 1 mM phenylmethanesulfonyl fluoride; 1 mM *p*-(hydroxymercuri)benzoic acid sodium salt; 5 mM benzamidine hydrochloride; 50 mM ϵ-aminocaproic acid. The suspension was centrifuged every 48 h (Sorval RC-5B, SA-600 rotor, 10000 rpm, 30 min), the EDTA-soluble extracts were decanted and frozen at −20 °C, and the insoluble residues were reextracted with additional EDTA and inhibitors. The extraction process was discontinued after the calcium ion concentration of two successive EDTA extracts was less than 10^{-6} M. The final insoluble residue, composed principally of collagen, was washed 3 times with 10 volumes of distilled water, 4 °C, and lyophilized. The pooled EDTA extracts were desalted and concentrated by diafiltration (Lee & Glimcher, 1981) with 0.05 M NH_4HCO_3, pH 7.9 The same protease inhibitors were present. The extracts were then lyophilized.

Sephadex G-100 Chromatography. EDTA extracts were first fractionated by molecular sieve chromatography on a 2.2 cm × 200 cm column of Sephadex G-100 equilibrated in and eluted by 0.1 M NH_4HCO_3, pH 8.8, at 4 °C. Each 5-mL fraction was read manually for absorbance at 230 nm, and 100-μL aliquots of each fraction were analyzed for organic phosphorus (Lee, 1977; Lee et al., 1977). Molecular sieving resolved macromolecular components from low molecular weight species (EDTA, inorganic orthophosphate, inhibitors) which eluted as a broad peak after fraction 120 [Figure 1 (peak not shown)]. Molecular weight markers are explained in the legend of Figure 1. Pooled G-100 fractions were lyophilized, weighed, and analyzed for organic phosphorus, protein, uronic

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Gdn·HCl, guanidine hydrochloride.

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acid, and amino acid composition.

DEAE-cellulose Chromatography. Phosphorus-containing G-100 fractions were further fractionated individually by anion-exchange chromatography at 4 °C. One hundred milligrams of the G-100 fractions dissolved in 10 mL of 0.05 M Tris-HCl, pH 8.2, was applied to 2.5 cm × 10.5 cm columns of DEAE-cellulose (Whatman DE-52) in the same buffer. Proteins were eluted by a linear NaCl gradient (0–0.5 M) in the same buffer and were detected by manual reading of absorbance at 230 nm. The salt gradient was monitored by conductivity measurements. Aliquots of each fraction were analyzed for phosphorus (Lee et al., 1977) and for uronic acid contents (Bitter & Muir, 1962). Phosphoprotein-enriched fractions were pooled and dialyzed against water (Spectrapor-3, Spectrum Medical Industries) and lyophilized.

Sephacryl S-300 Chromatography. Phosphoprotein-enriched pools from DEAE-cellulose chromatography were individually analyzed for molecular weight distribution by molecular sieve chromatography in 6 M guanidine hydrochloride and 0.02 M Tris-HCl, pH 8.5, on Sephacryl S-300 (1.6 cm × 180 cm; Pharmacia). Each sample was reduced and alkylated in the same solvent prior to chromatography (Lee et al., 1977). Sephacryl S-300 fractions were read manually for absorbance at 230 nm, pooled, dialyzed, and lyophilized. The following proteins, of known molecular weight, were similarly reduced, alkylated, and filtered: phosphorylase *b* subunit (M_r 94 000); serum albumin (M_r 66 000); catalase subunit (M_r 57 000); ovalbumin (M_r 45 000); phosvitin (M_r 36 000); lysozyme (M_r 14 400). Distribution coefficients, K_{av}, were calculated as $K_{av} = (V_e - V_o)/(V_i - V_o)$, where V_o and V_i were the elution volumes for Blue Dextran and *N*-(dinitrophenyl)-L-isoleucine, respectively. It should be noted that the manufacturer's reported exclusion limit of 1.5×10^6 was markedly reduced to approximately 1×10^5 in this solvent.

Amino Acid Analysis. Hydrolyses for amino acid analyses were carried out in vacuo in three ways: limited acid hydrolysis (4 N HCl, 106 °C, 4–6 h) for Ser(P) and Thr(P); total acid hydrolysis (6 N HCl, 106 °C, 22 h) for standard amino acid analysis; base hydrolysis (2 N KOH, 100 °C, 22 h) for determination of γ-carboxyglutamic acid. Amino acid contents were determined on the Beckman 121-M single column analyzer by using three programs: one for the standard amino acids, one which resolved Ser(P), Thr(P), cysteic acid, and taurine (Cohen-Solal et al., 1978), and one which resolved γ-carboxyglutamic acid from aspartic acid (Hauschka, 1977). No corrections for hydrolytic losses of serine or Ser(P) were used. Calculations of residues per 1000 residues were the arithmetic averages of triplicate or quadruplicate analyses and were reproducible to ±5% of the mean.

Results

Chemical Characterization of Phosphoproteins from Fetal and Adult Molar Dentin. Amino acid compositions of the EDTA-extractable proteins from fetal and adult dentin and their respective demineralized insoluble residues are shown in Table I. The phosphoserine remaining in the residues after EDTA extraction was not removed by further prolonged EDTA extraction or by 6 M guanidine hydrochloride extraction. At least part of this insoluble phosphoprotein represents a fraction which is very closely associated with and possibly covalently linked to the dentin collagen (Lee & Veis, 1980; Curley-Joseph & Veis, 1979). Furthermore, the Ser(P) content of the EDTA- and Gdn·HCl-insoluble residue of steer dentin was approximately twice that of fetal dentin, and there were small but detectable amounts of Thr(P) in the EDTA-insoluble residue of steer dentin, but not in the EDTA-insoluble residue of fetal dentin. The presence of phosphoprotein in dentin which is insoluble in EDTA and Gdn·HCl has been previously reported (Kuboki et al., 1979; Lee & Veis, 1980; Curley-Joseph & Veis, 1979). We would therefore like to make it clear that the results obtained in this study refer only to that fraction of phosphoprotein which is soluble in EDTA and, when solubilized, is not complexed or covalently bound to collagen. Further studies on the EDTA- and Gdn·HCl-insoluble fraction of the phosphoproteins in dentin and bone are currently under way and will be reported separately.

Table I: Amino Acid Compositions of EDTA Extracts and EDTA-Insoluble Residues of Fetal and 2-Year-Old Steer Molar Dentin

	residues/1000 residues			
	fetal extract	2-year-old extract	fetal residue	2-year-old residue
Cyt	30	3		
Hyp	0	44	101	104
Asp	228	158	53	58
Thr	31	25	17	16
Ser[a]	204	117	38	43
Glu	91	80	72	75
Pro	55	107	109	104
Gly	71	186	340	330
Ala	45	72	106	106
Cys	12	9		
Val	32	30	19	20
Met	7.5	4.6	5.5	5.4
Ile	25	15	11	11
Leu	44	34	25	25
Tyr	14	9.8	3.2	3.4
Phe	19	15	14	14
His	18	12	5.2	6.3
Hyl	0	6.1	10	13
Lys	56	37	23	22
Arg	23	35	50	46
Gla	0.9	1.3	0	0
Ser(P)[b]	82	58	2.1	4.8
Thr(P)[b]	0	0	0	trace

[a] Includes Ser(P). [b] Determined by modified program for analysis on Beckman 121M of a 6-h acid hydrolysate: 4 M HCl, 106 °C. Value uncorrected for degradation.

There were several significant differences in the composition of the EDTA-soluble proteins from fetal and steer dentin (Table I). The EDTA-soluble proteins from fetal dentin were considerably richer in aspartic acid, serine, and Ser(P) but contained considerably less hydroxyproline and slightly less γ-carboxyglutamic acid than the EDTA-soluble proteins from steer dentin, demonstrating that there was more phosphoprotein in the EDTA-soluble extract of fetal dentin than in the EDTA extract of steer dentin.

Sephadex G-100 Chromatography of EDTA Extracts. Molecular sieving of EDTA extracts of fetal and steer dentin on Sephadex G-100 in nondenaturing buffer is shown in Figure 1. The elution profiles for fetal and steer EDTA extracts were qualitatively similar. Both showed a major phosphorus-enriched, excluded peak (fractions 45–65), smaller amounts of organic phosphorus in included components (fractions 66–120), and broad, intensely absorbing salt peaks (after fraction 120, not shown). However, two important differences were noted: (1) the protein to phosphorus ratio was higher for the G-100 excluded peak of steer dentin, and (2) there were more low molecular weight, phosphorylated components in steer extracts than in the fetal extracts. The histogram insets in Figure 1 illustrating the effluent fraction pools (A–D), extend the first observation: the phosphoprotein content of G-100 pool A of fetal dentin extract (i.e., the excluded peak) is significantly higher than that of the comparable steer fraction.

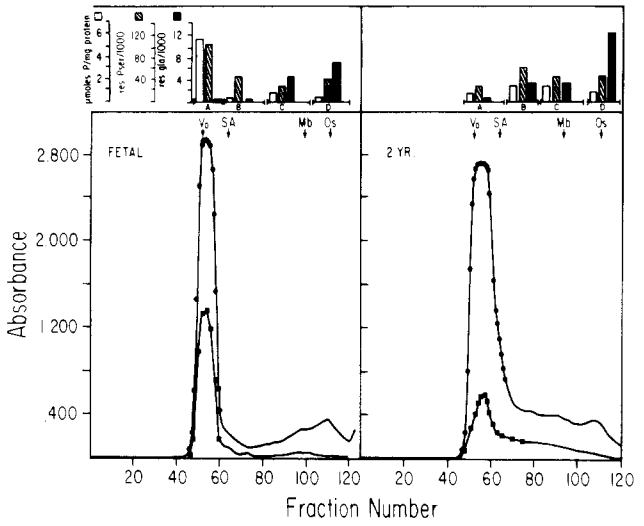

FIGURE 1: EDTA extracts of fetal and adult dentin are first fractionated by gel filtration on Sephadex G-100 in 0.1 M NH_4HCO_3, pH 8.8, at 4 °C. The absorbance at 230 nm (●), of each fraction, was measured manually. Aliquots of each fraction were wet ashed and analyzed for organic phosphorus, and the absorbance at 830 nm (phosphomolybdate) was measured. Arrows indicate elution peaks for molecular weight markers: V_0 (Blue Dextran), SA (bovine serum albumin, M_r 66 000), Mb (horse myoglobin, M_r 16 950), and Os (chicken bone osteocalcin, M_r 6000). Histogram insets demonstrate the method for pooling effluent fractions (A–D) and summarize analytical data for these pools: residues of γ-carboxyglutamic acid per 1000 residues, residues of phosphoserine per 1000 residues, and micromoles of organic phosphorus per milligram of Lowry protein.

Table II: Distribution of Phosphorus, Protein, and Uronic Acid in Sephadex G-100 Fractions

G-100 fractions	% (w/w) of dry wt	μmol of P/mg of protein	recovered organic P (%)	recovered protein (%)	μg of uronic acid/mg of protein
		Fetal Dentin			
A	74	5.66	97	70	63
B	13	0.16	0.6	14	0
C	9	0.73	1.7	10	0
D	4	0.38	0.6	6	0
total	100	4.05	100	100	63
		Steer Dentin			
A	65	0.69	55	70	35
B	15	1.38	19	12	0
C	14	1.4	20	13	0
D	6	0.84	5	6	0
total	100	0.87	100	100	35

Table II summarizes the distribution of phosphorus, protein, amino acids, and uronic acids in each of the G-100 pooled fractions from steer and fetal EDTA extracts. Nearly all (97%) of the organic phosphorus in the fetal EDTA extract is recovered in the excluded peak (pool A), whereas only 55% of the organic phosphorus of the EDTA extract of steer dentin is recovered in the excluded peak. The remaining 45% of the organic phosphorus is unequally distributed among the three low molecular weight pools (B–D). From Figure 1 and Tables I and II, it may be concluded that phosphoproteins represent a larger fraction of the EDTA-extractable proteins of fetal dentin than they do of the EDTA-extractable proteins of steer dentin. Moreover, there are more low molecular weight phosphoprotein components in the EDTA extracts of steer dentin than in the EDTA extracts of fetal dentin.

Quantitative Isolation and Purification of Phosphoproteins by DEAE-cellulose and Sephacryl Chromatography. Phosphoproteins from steer and fetal dentin were separately purified

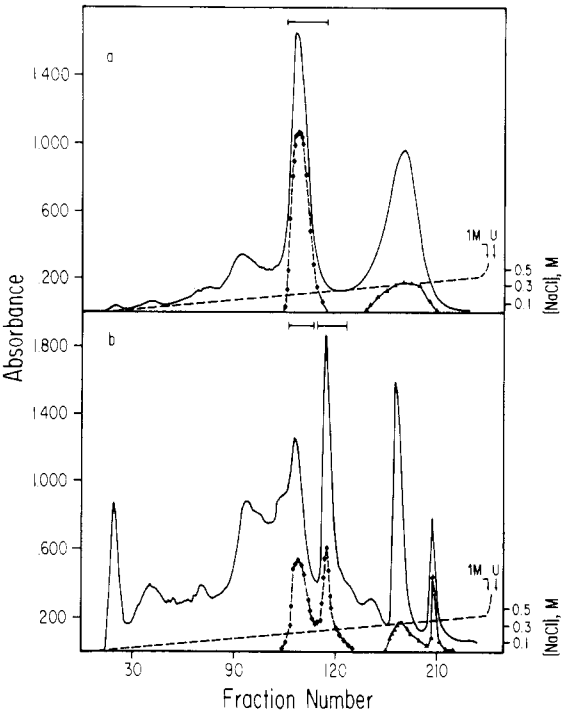

FIGURE 2: Anion-exchange chromatography (DEAE-cellulose) at pH 8.2, 4 °C, of the Sephadex G-100 void volume peaks from extracts of fetal (a) and steer (b) dentin (see Figure 1). Protein elution by the linear NaCl gradient (---) was detected by measuring the 230-nm absorbance (—) of each fraction. Aliquots of each fraction were analyzed for phosphorus [830-nm absorbance (♦)] and for uronic acid [530-nm absorbance (▲)]. Arrows indicate that 1 M NaCl alone (1 M) or with 6 M urea (U) failed to elute additional components. Fractions under bars were pooled for analysis and molecular sieving (see Figure 3).

from their respective G-100 pool A (see Figure 1) by DEAE-cellulose chromatography (Figure 2). By use of a shallow NaCl gradient, similar to that of Dimuzio & Veis (1978), only one phosphoprotein peak, eluting at 0.21–0.24 M NaCl, was resolved from G-100 pool A of fetal dentin (Figure 2a). The single phosphoprotein peak in Figure 2a accounted for all of the applied phosphorus. Its amino acid composition was characterized by an aspartic acid content of 372 residues/1000 total amino acid residues and serine and Ser(P) of 400 residues/1000 total amino acid residues. In contrast, identical anion-exchange chromatography of fraction A from the G-100 sieving of steer dentin (Figure 2b) showed two phosphoprotein fractions which differed from the phosphoproteins isolated from embryonic dentin in their protein to phosphorus ratios, in their affinities for DEAE-cellulose, in the fraction of the total phosphorus (60% and 40%), and in amino acid composition (254 residues of aspartic acid and 238 residues of serine plus phosphoserine per 1000 residues, compared with 267 residues of aspartic acid and 200 residues of serine plus phosphoserine per 1000 residues). Figure 2b shows that unlike the EDTA-soluble phosphoprotein from fetal dentin, the EDTA-soluble phosphoproteins of steer dentin are heterogeneous.

To further isolate the EDTA-soluble phosphoproteins of fetal and steer dentin and to simultaneously estimate their molecular weights, each phosphoprotein fraction (bars in Figure 2) was separately analyzed by molecular sieving under denaturing conditions. Figure 3a shows the elution profile of fetal phosphoprotein from Sephacryl S-300 chromatography in 6 M Gdn·HCl. Sephacryl S-300 chromatography in 6 M Gdn·HCl yielded a linear molecular weight plot for five proteins, including three phosphoproteins (Figure 4).

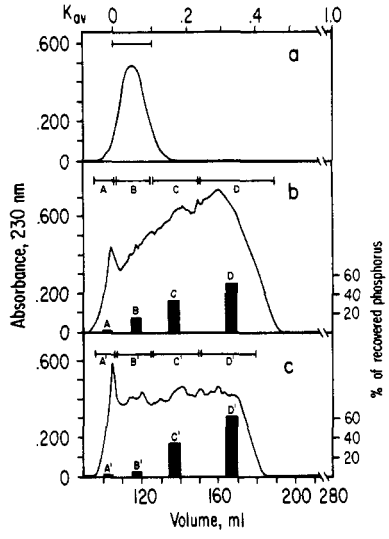

FIGURE 3: Molecular sieving on Sephacryl S-300 in 6 M guanidine hyrochloride–0.02 M Tris, pH 8.5, of the phosphoprotein-enriched fractions obtained by anion-exchange chromatography (Figure 2). Samples were reduced and alkylated prior to chromatography, and protein elution was detected by 230-nm absorbance. Effluent fractions, pooled as indicated by the bars, were analyzed for phosphorus content (see histogram insets) and amino acid composition (Table IV). (a) Elution profile for the single fetal phosphoprotein peak obtained in Figure 2a; (b) elution profile for the adult phosphoprotein peak eluting at 0.18–0.22 M NaCl in Figure 2b; (c) elution profile for the adult phosphoprotein peak eluting at 0.22–0.25 M NaCl in Figure 2b.

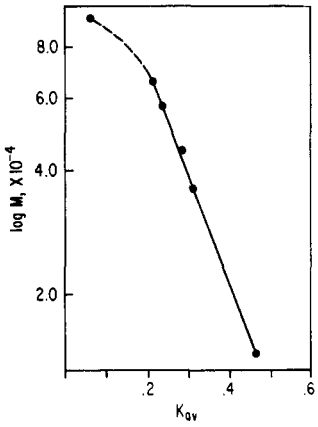

FIGURE 4: Molecular weight calibration curve for Sephacryl S-300 chromatography in 6 M guanidine hydrochloride. Purified proteins of known molecular weight were reduced and alkylated prior to chromatography. See Materials and Methods for details.

Fetal phosphoprotein (Figure 3a) appeared monodisperse and had a K_{av} of 0.055, identical with that of rabbit muscle phosphorylase *b* (94 000 daltons). Both before and after chromatography in 6 M Gdn·HCl, fetal phosphoprotein contained 8% (w/w) phosphorus. This single symmetrical peak accounted for all of the applied phosphorus, and the only phosphorylated amino acid detected was Ser(P). The amino acid composition (Table III, column 1) was invariant across the peak. The results are in good agreement with the results of Termine et al. (1980) for the phosphoprotein of fetal calf dentin extracted by solutions of Gdn·HCl and guanidine hydrochloride–EDTA.

In contrast to the EDTA-extractable phosphoprotein of fetal dentin, the EDTA-extractable phosphoproteins of steer dentin were heterogeneous. When the two phosphoprotein fractions of steer dentin (Figure 2b) were analyzed separately for molecular weight distributions (Figure 3b,c), both preparations were polydisperse, spanning the K_{av} range 0–0.4 or a molecular weight range of 21 000 to more than 94 000. Approximate

Table III: Amino Acid Compositions of Sephacryl S-300 Fractions of Fetal and Steer, EDTA-Extracted Phosphoproteins

	residues/1000 residues					
		steer				
	fetal 0.055[a]	0–0.1[a]	0.1–0.24[a]	0.25–0.35[a]	0.35–0.38[a]	0.39–0.45[a]
Cyt[b]	25	31	43	43	23	27
Hyp	0	3.1	5.6	8.1	0	0
Asp	371	169	238	303	359	388
Thr	8	45	34	23	17	9
Ser[c]	405	151	217	286	277	303
Glu	27	108	84	69	70	49
Pro	6.5	56	42	34	23	20
Gly	54	85	74	67	80	77
Ala	14	64	44	28	23	20
Cys	0	0	0	0	10	13
Val	13	52	33	19	12	9
Met	0	0	3.0	2.0	1	1
Ile	3.7	24	18	11	8	4
Leu	6.4	55	39	20	15	11
Tyr	1.0	14	13	5.6	6	6
Phe	2.1	25	17	7.9	6	4
His	7.5	20	16	11	11	10
Hyl	0	0	0.7	1.0	0	0
Lys	55	63	56	49	44	41
Arg	0	32	25	15	15	8
Ser(P)[d]	240	53	102	145	114	129
Thr(P)[d]	0	0	0.1	0.13	0	0

[a] K_{av} on Sephacryl S-300. [b] Includes Ser(P), Thr(P), and Cyt in 22-h acid hydrolysate. [c] Values are direct analysis values for 22-h acid hydrolysates and are not corrected for hydrolytic losses of Ser or Ser(P). [d] Quantified by a modified analyzer program for acidic amino acids only in 4-h acid hydrolysates.

effluent fractions were pooled to represent the excluded volume (A and A′), the fraction corresponding to fetal phosphoprotein (B and B′), and two lower molecular weight pools (C, C′, D and D′). Only a small fraction of the effluent phosphorus (15% and 6%, respectively) was actually recovered in the K_{av} region 0–0.1, whereas more than 80% of the phosphorus eluted in two lower molecular weight effluent pools, K_{av} = 0.1–0.43, spanning the molecular weight range 21 000 to less than 94 000. Amino acid analysis of these effluent pools from Sephacryl chromatography (Figure 3c and Table III, columns 2 and 4) showed that (1) the steer phosphoprotein pools were heterogeneous, (2) the amounts of phosphorus-free protein were variable, being largest for the K_{av} = 0–0.1 pools, and (3) hydroxyproline- and Thr(P)-containing proteins were more abundant in the lower molecular weight region. Taken together, Figure 3 and Table III establish that the highest molecular weight phosphoproteins of steer dentin are a family of aspartic acid and Ser(P)-rich proteins ranging in molecular weight from 21 000 to 87 000.

Further evidence for steer dentin phosphoprotein heterogeneity was obtained by isolating the lower molecular weight phosphorylated components in G-100 pools B and C of steer dentin (see Figure 1 and Table II). Figure 5a shows protein and phosphorus elution profiles from DEAE-cellulose chromatography for combined steer G-100 pools B and C. Coeluting in a large unsymmetric peak at 0.2–0.25 M NaCl were at least three phosphorylated proteins. When this entire phosphorus-containing peak (see bar in Figure 5a) was filtered on Sephacryl S-300 in 6 M Gdn·HCl (Figure 5b), marked molecular weight heterogeneity was seen in the K_{av} region of 0.34–0.43, or 18 000–35 000 daltons. Amino acid analyses of these Sephacryl fractions (see bars in Figure 5b and compositions in Table III, columns 5 and 6) confirmed the presence of aspartic acid–Ser(P)-enriched phosphoproteins in these low molecular weight fractions.

Table IV: Molecular Weight Distributions of Phosphoproteins from Fetal and Adult (Steer) Bovine Dentin

	fetal	steer				
	0.01[a]	0–0.1[a]	0.1–0.24[a]	0.24–0.34[a]	0.34–0.38[a]	>0.38[a]
molecular weight range	≥94 000	87 000–94 000	54 000–87 000	35 000–54 000	21 000–35 000	<21 000
total phosphoprotein[b] (%)	≈100	2.2	19	34	20	26

[a] K_{av} on Sephacryl S-300. [b] Based on phosphorus and dry weight recoveries.

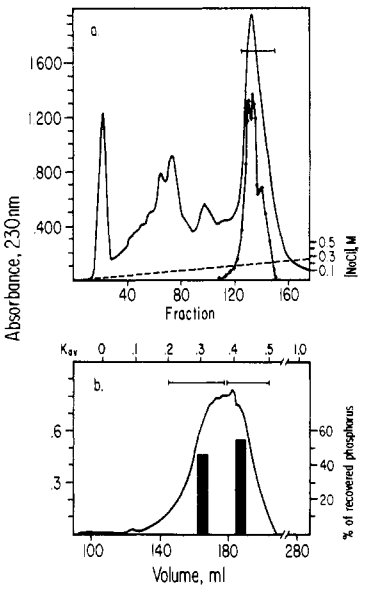

FIGURE 5: (a) Anion-exchange chromatography (DEAE-cellulose) at pH 8.2, 4 °C, of the pooled adult Sephadex G-100 fractions B and C (see Figure 1). Elution and detection of proteins (—) and of organic phosphorus (♦) were identical with those in Figure 2. Fractions under the bar were pooled for molecular sieving on Sephacryl S-300 (below). (b) Molecular sieving on Sephacryl S-300 in 6 M guanidine hydrochloride–0.02 M Tris, pH 8.5, of the adult phosphoprotein fraction obtained by anion-exchange chromatography in (a). Protein elution was detected at 230 nm (—), and fractions under the bar were pooled and analyzed for phosphorus content (histogram) and amino acid content (Table IV).

Since phosphorus recovery from each cycle of chromatography and dialysis was quantitative (from 90 to 95%), it was possible to calculate the molecular weight distribution for all phosphoproteins from steer dentin from the phosphorus recovery data (Table IV). The majority of the phosphoproteins from steer dentin (54%) ranged in molecular weight from 21 000 to 54 000. Very little of the high molecular weight phosphoprotein found in fetal dentin was present in the EDTA-soluble proteins of steer dentin.

In order to rule out the possibility that the polydispersity of the phosphoproteins of mature dentin was the result of in vitro degradation due to enzyme contamination, EDTA extracts of both embryonic dentin (older than the 3–4-month-old fetuses used in the original experiments, i.e., 8–9-month-old embryos) and mature dentin containing the full complement of protease inhibitors were dialyzed but incompletely free of EDTA, lyophilized, redissolved in 6 M Gdn·HCl, reduced, and molecularly sieved directly in 6 M Gdn·HCl on Sephacryl S-300 and high-precision liquid chromatographed, through two TSK-4000 columns in tandem (Beckman Instruments, Berkeley, CA). On Sephacryl S-300, approximately 60% of the applied Ser(P) in the embryonic dentin extract was eluted in fractions having a molecular weight of ≈90 000 or greater, while approximately 27% was eluted in fractions with a molecular weight of ≈25 000 and approximately 13% with a molecular weight of ≈12 000 or less. Comparable values for the extract obtained from mature dentin were approximately 8% of the Ser(P) recovered in molecular weight fractions of ≈90 000 and above, approximately 28% in molecular weight fractions of ≈25 000, and approximately 54% in molecular weight fractions of ≈12 000 or less. By HPLC, approximately 59% of the Ser(P) of the embryonic dentin extract compared with 15% of the mature dentin extract was eluted in the molecular weight range of ≈94 000 and higher, while 41% of the Ser(P) of the embryonic dentin extract compared to approximately 85% of the Ser(P) of the adult dentin extract was eluted in the molecular weight range of 24 000 and below.

These results, obtained from EDTA extracts which from the time of extraction from the tissue to and including the period of molecular sieving contained enzyme inhibitors, effectively rule out the possibility that the polydispersity of the phosphoproteins of mature dentin occurred in vitro and establish that breakdown of the phosphoproteins occurs in vivo with maturation. It is quite interesting and informative that significant degradation of the M_r ≈94 000 phosphoprotein has occurred in the 8–9-month-old embryonic dentin, compared to the dentin of 3–4-month-old embryos. Since dentin, unlike bone, is not continuously being remodeled (viz., mature tissue is not replaced by newly synthesized tissue), the age of the animal is a very good measure of the age of its structural components. Thus, the data on the distribution of molecular species by weight in the two groups of embryos are good indicators of the rate at which the high molecular weight phosphoprotein is degraded and probably explain why the average molecular weight of the principal phosphoprotein found in young postnatal calves was ≈35 000 (Lee et al., 1977).

Although the highest molecular weight phosphoprotein isolated from very young fetal dentin was a single homogeneous component of approximately 94 000, it is conceivable that higher molecular weight species exist intracellularly and/or extracellularly in more immature and more recently synthesized dentin matrix or as covalent complexes with collagen.

Our final observation concerned the glycoaminoglycans in the EDTA extracts. All of the uronic acid containing species in the EDTA extracts of both steer and fetal dentin were macromolecular, eluting only in the excluded volumes from Sephadex G-100 filtrations (Figure 1 and Table II). However, the uronic acid elution profiles from DEAE-cellulose chromatography of each G-100 excluded peak (Figure 2) were clearly distinctive. The glycosaminoglycans of fetal dentin were quantitatively eluted as a broad peak at 0.32–0.36 M NaCl, whereas those from steer dentin eluted as major and minor peaks at 0.32 and 0.38–0.4 M NaCl. Further studies on their compositions and molecular weights are in progress.

Discussion

Previous studies on dentin phosphoproteins have reported molecular weights ranging from approximately 30 000 to 100 000 (Veis et al., 1972; Lee et al., 1977; Jontell & Linde, 1977; Termine et al., 1980; Linde et al., 1980). Part of the discrepancy has been ascribed to species differences (rat vs. bovine). However, even within the bovine species, phosphoproteins with molecular weights of 35 000–40 000 (Lee et al., 1977) and of nearly 100 000 (Termine et al., 1980) have been isolated with similar amino acid compositions. Although it

is possible that these data represent degradation of the phosphoproteins during isolation and preparation, it is also possible that it reflects degradation during in vivo maturation (Dimuzio & Veis, 1978; Veis et al., 1981).

The present experiments, characterizing the phosphoproteins extracted from very young, fetal calf dentin and from mature, adult bovine dentin under identical conditions designed to eliminate or minimize proteolysis, strongly suggest that the single high molecular weight phosphoprotein of fetal calf dentin is degraded during the progressive mineralization of dentin during maturation. This may at least partially explain the earlier finding that the major phosphoprotein of young steer dentin was much smaller and had a much lower phosphorus content (4.8% vs. 8.0%).

Degradation of the phosphoproteins of mineralized tissues during maturation appears to be a general phenomenon. Direct evidence for the in vivo degradation of the enamel proteins has been obtained (Strawich & Glimcher, 1982), and there is highly suggestive evidence that the phosphoproteins of bone are also degraded during maturation (Lee & Glimcher, 1979, 1981).

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Sequence-Specific Interaction of R17 Coat Protein with Its Ribonucleic Acid Binding Site†

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ABSTRACT: The interaction between phage R17 coat protein and its RNA binding site for translational repression was studied as an example of a sequence-specific RNA–protein interaction. Nuclease protection and selection experiments define the binding site to about 20 contiguous nucleotides which form a hairpin. A nitrocellulose filter retention assay is used to show that the binding between the coat protein and a synthetic 21-nucleotide RNA fragment conforms to a simple bimolecular reaction. Unit stoichiometry and a K_d of about 1 nM are obtained at 2 °C in buffer containing 0.19 M salt. The interaction is highly sequence specific since a variety of RNAs failed to compete with the 21-nucleotide fragment for coat protein binding.

Efforts to understand the molecular basis of the specific interaction between an RNA sequence and a protein are often hampered by the complexity of the system. For example, in the case of multistep catalytic reactions such as the aminoacylation of tRNAs by synthetases, it may be difficult to provide a simple description of the reaction in terms of individual contacts on the surfaces of both RNA and protein molecules. This consideration prompted us to search for a sequence-specific RNA–protein interaction in which biological function is exerted in a single binding event. Such a system should permit description of the reaction as a simple bimolecular equilibrium amenable to detailed physical and biochemical characterization. The success of this approach in understanding sequence-specific DNA–protein interactions is exemplified by work on the *lac* repressor (Riggs et al., 1970; Record et al., 1977; Butler et al., 1977; Barkley et al., 1981; Winter & von Hippel, 1981).

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