Studies of the Proteins, Peptides and Free Amino Acids of Mature Bovine Enamel

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1. The organic matrix of enamel from erupted bovine teeth has been found to be composed mostly of small peptides containing principally aspartic acid, glycine, glutamic acid and serine. 2. A small amount of higher-molecular-weight components has been isolated by various procedures. One non-diffusible fraction was found to be heterogeneous in the ultracentrifuge, and composed principally of material that by gel filtration indicated a molecular weight greater than 30 000. These components were largely carbohydrate in nature (glycoproteins and glycopeptides), containing only small amounts of amino acids.

Previous studies have described several methods for the isolation of the soluble and insoluble components of the organic matrix of mature bovine enamel essentially free from contamination with either dentinal or cemental collagen (Glimcher, Friberg & Levine, 1964c). The amino acid composition of the whole organic matrix, as well as of a number of soluble and insoluble fractions, differed markedly from that of both collagen and proteins of embryonic enamel (Glimcher *et al.* 1964c; Eastoe, 1960, 1963; Piez, 1961; Glimcher, Mechanic, Bonar & Daniel, 1961; Glimcher, Mechanic & Friberg, 1964b). Further, in contrast with the proteins of embryonic bovine enamel, most of the amino acid-containing constituents were readily diffusible through dialysis sacs or retarded on columns of Sephadex G-25 resin (Glimcher *et al.* 1964c). These findings, coupled with the fact that over 90% of the enamel proteins was lost during the development of the tooth, suggested that maturation of the enamel was accompanied by degradation of the matrix proteins, especially those components rich in proline and histidine but poor in serine (Glimcher *et al.* 1964b,c). The present paper deals with the further characterization of the proteins, peptides and free amino acid constituents of the organic matrix of enamel from erupted mature bovine teeth.

EXPERIMENTAL

Tissue source. The four central permanent erupted incisor teeth of 24–40-month-old steers were used. After dissection from the lower jaw, the crown of the tooth was separated from the root by sectioning the tooth approx. 3–5 mm. above the gingival margin, and the pulp chambers

were cleaned. Very heavily stained, cracked or pitted teeth were discarded.

Isolation of the organic constituents of the enamel from selected areas of the labial surface of the tooth crown. The tooth crowns were placed in 0.5 M-EDTA, pH 8.3, at 2° or 25° for 48 hr. with toluene added for bacteriostasis. At the end of this period, the coronal cementum (Glimcher, Friberg & Levine, 1964d) was dissected free from the underlying enamel, and the tooth surface brushed gently. The tooth crowns were again placed in 0.5 M-EDTA, pH 8.3, for approx. 4 hr., their surfaces brushed briskly in EDTA, and rinsed in double-distilled demineralized water. After they were dried at 25° over P₂O₅ in vacuum desiccators, the pulp chambers were filled with dental acrylic resin and all but a 1.0 cm. × 1.0 cm. area of the central portion of the labial surface of the enamel was covered with (finger) nail varnish (Cutex; Chesebrough-Ponds Inc., New York, N.Y., U.S.A.) (Glimcher *et al.* 1964c).

Two methods were used to dissolve the mineral phase (and most of the organic matrix) of the selected area of crown enamel. In the first method, the tooth crowns, prepared as described, were placed in covered polystyrene trays containing 0.5 M-EDTA, pH 8.0, at 8–10°. The EDTA solution was circulated continuously by means of a peristaltic pump and changed at 48 hr. intervals. The teeth were carefully observed during the procedure, and coronal and cross-sections made of randomly selected teeth so that the procedure could be terminated while an adequate thickness of undecalcified enamel still remained covering the dentine over the exposed labial surface. All the EDTA solutions were pooled and analysed as a single sample. About 5000 teeth were used for this experiment. In the second procedure, the tooth crowns were placed in the polystyrene trays and covered with double-distilled demineralized water. Then HCl gas was bubbled in at a constant rate to maintain the pH at 2.3–2.8 and the solution circulated by means of a peristaltic pump. About 2000 teeth were used in this experiment.

A third method was used to obtain an ethanol-insoluble

Scheme 2. Procedures employed in the chromatographic separation of the peptides and free amino acids of EDTA-soluble fraction B. The numbers in parentheses refer to the columns in Table 2.

fraction of the organic matrix. In this technique, whole tooth crowns were placed in EDTA and the coronal cementum was removed as described. The crowns were then placed in fresh 0.5M-EDTA, pH 8.3, at 2° for 4–6 hr., during which time the surfaces were brushed briskly several times. The tooth crowns were removed from the EDTA solution, washed thoroughly with water and dried. The labial surface of the tooth crowns was then stained with haematoxylin, which was found to be a very sensitive index of the presence of small remnants of the cementum. If there was positive-staining material present the crowns were placed for various times in ethanol to which was added conc. HCl to give a final concn. of 0.1 N, and the softened surface layer was scraped gently with a sharp scalpel blade. The procedure was repeated until the surface of the enamel did not stain with haematoxylin. The crowns were then cut into 1000 μ -thick cross-sections on a modified Gillings-Hamco apparatus (Hamco Machine Co. Inc., Rochester, N.Y., U.S.A.) and slabs of enamel dissected free from the dentine by an air-abrasive technique (Friberg, Levine & Glimcher, 1964). The surface of the enamel slabs closest to the dentino-enamel junction were then further abraded by using dental brushes.

The enamel slabs were placed in ethanol made 0.1 N with respect to HCl at room temperature and the solution was stirred gently. The decalcified precipitate was separated by centrifugation, washed with ethanol made 0.1 N with respect to HCl and dried. Except for amino acid analyses, this material was not further utilized in this study.

Recovery and fractionation of the EDTA- and hydrochloric acid-soluble proteins, peptides and amino acids. The procedures followed are outlined in Schemes 1, 2 and 3. The EDTA solutions were filtered or centrifuged to obtain the EDTA-insoluble residue and a clear supernatant. The EDTA-insoluble residue, representing only a very small portion of the organic matrix, was washed quickly with water, and then placed in water adjusted to pH 2.0 with HCl at 2° and stirred. After 48 hr. the insoluble residue (EDTA-insoluble acid-insoluble fraction) was separated from the supernatant (EDTA-insoluble acid-soluble fraction). Both fractions were used only for amino acid analysis.

Most of the EDTA was precipitated from the original clear supernatant (EDTA-soluble fraction; Scheme 1) by adjusting the pH to 1.5 at 2°. The clear supernatant was concentrated on a rotary evaporator, and the procedure repeated several times (Glimcher *et al.* 1964c). The final supernatant was adjusted to pH 2.0–2.5 and divided equally into two portions. One half of the solution was dialysed exhaustively against water adjusted to approx. pH 7.0 with aq. NH₃. The soluble and insoluble fractions of this non-diffusible fraction were separated by centrifugation. The insoluble residue was used only for amino acid analysis. The water-soluble fraction was freeze-dried and a portion used for chemical analyses. The remainder of this fraction was dissolved (with difficulty) in 6M-urea in 0.15M-tris-HCl buffer, pH 7.4. A portion of the material soluble in 6M-urea was used for analytical ultracentrifugation studies, and the

remainder of the soluble portion filtered through a column (0.9 cm. $\times$ 48 cm.) of Bio-Gel P-30 resin (50-150 mesh) (copolymer of acrylamide and methylenebisacrylamide; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) equilibrated in 6M-urea. Two fractions were collected: one that was excluded from the column and therefore represented material of mol.wt. greater than 30000, and a second that was retarded on the column and therefore contained material of mol.wt. less than 30000. The urea was removed by trituration with ethanol and the material was dried. Because of the small amount of material in these fractions and its relative insolubility, no further physicochemical studies were undertaken.

The other half of the EDTA-soluble fraction was filtered through a column (3 cm. $\times$ 170 cm.) of Sephadex G-25 resin equilibrated with 5% acetic acid, pH 2.5, at 2°, and four fractions were collected. Bovine serum albumin, NaCl and the peptides glycyl-L-aspartic acid, glycyl-L-leucylglycine, tetraglycine and pentaglycine, as well as the amino acids glycine and aspartic acid, were used to standardize the column run under identical conditions.

Fraction B (see Scheme 1) was further subjected to the procedures outlined in Scheme 2. A portion was weighed out and hydrolysed in triple-distilled constant-boiling 6N-HCl for 24 hr. at 105°. The hydrolysate was evaporated to dryness three times and desalted on a column of Dowex 50 (X2; H⁺ form) resin, and subjected to amino acid analysis. This represented the total amino acid composition and

content of fraction B. The remainder of the material was weighed, dissolved in 0.01N-HCl and chromatographed on a column (2.4 cm. $\times$ 45 cm.) of Dowex 50 (X8; H⁺ form) resin. The sample was eluted with water and the first 4-6 column volumes eluted with water were separated from the material eluted thereafter with aq. N-NH₃. The percentage and composition of the material in the water and aq.-NH₃ fractions existing in peptide form and as free amino acids were determined by chromatographing unhydrolysed and hydrolysed portions on the automatic amino acid analyser.

The HCl-soluble material was separated as shown in Scheme 3. No HCl-insoluble precipitate was recovered. In this case most of the calcium and phosphate was removed before filtration through Sephadex G-25 by the addition of calcium acetate. Fractions C and D of the HCl-soluble material obtained by Sephadex G-25 filtration were further fractionated in the manner described for fraction B (Scheme 2). To assess the possible hydrolysis of peptides on the Dowex 50 (X8; H⁺ form) resin, known amounts of several peptides (e.g. glycyl-L-aspartic acid and glycyl-L-leucylglycine) were chromatographed on the same column under conditions identical with those used for the enamel peptides. The material eluted from the columns was then analysed directly in the automatic amino acid analyser without further hydrolysis to determine the amounts, if any, of free amino acids liberated.

To minimize the effect of peptide-bond hydrolysis that might have occurred during the repeated precipitations of

Scheme 3. Procedures employed for the isolation and separation of the HCl-soluble proteins and peptides of erupted bovine enamel.

Table 1. *Amino acid compositions of the EDTA-soluble and -insoluble components of the organic matrix of erupted bovine dental enamel*

The numbers at the top of the columns refer to the fractions shown in Scheme 1. Values are expressed as residues/1000 total amino acid residues.

	EDTA-soluble										Non-diffusible									
	EDTA-insoluble					Filtered through Sephadex G-25 resin					Soluble in 6M-urea					Water-soluble§ Filtered through Sephadex G-25 resin				
	Acid-insoluble		Fraction			Water-insoluble		Water-soluble			Total		Mol.wt.		Fraction	A		B		Ethanol-insoluble
	(1)	(2)	A	B	C	D	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)		(12)	(13)	(14)	(15)	
			(3)	(4)	(5)	(6)														
Cystine (half*)	3	—	—	<1	—	—	—	—	—	7	8	—	—	—	—	—	—	—	—	10
3-Hydroxyproline	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4-Hydroxyproline	58	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5
Aspartic acid	37	343	153	85	70	44	266	223	140	148	99	82	66	215	—	82	54	43	35	215
Threonine	11	19	90	31	15	12	26	37	36	36	35	37	35	35	—	37	35	35	35	35
Serine	20	345	127	64	51	57	186	167	130	126	148	199	258	201	—	199	199	258	201	201
Glutamic acid	58	30	150	129	134	71	100	141	124	129	99	197	138	77	—	197	138	77	77	77
Proline	214	21	78	28	8	25	33	42	44	52	Trace	56	28	42	—	56	28	42	42	42
Glycine	248	142	161	511	573	459	153	123	190	189	197	152	227	115	—	152	227	115	115	115
Alanine	82	21	†	58	31	36	51	42	67	68	63	92	82	73	—	92	82	73	73	73
Valine	46	10	60	27	14	17	21	58	39	35	42	40	26	36	—	40	26	36	36	36
Methionine†	<1	2	1	10	9	4	4	30	29	29	28	2	—	7	—	2	—	—	—	—
Isoleucine	32	†	22	†	†	16	13	28	29	29	28	23	15	20	—	23	15	20	20	20
Leucine	74	†	41	34	†	20	27	35	38	38	35	39	22	43	—	39	22	43	43	43
Tyrosine	11	Trace	2	4	82	Trace	Trace	Trace	3	Trace	21	6	10	5	—	6	10	5	5	5
Phenylalanine	28	5	14	8	Trace	179	15	14	23	20	35	15	24	22	—	15	24	22	22	22
Hydroxylysine	2	—	—	—	—	Trace	—	—	—	—	—	—	—	2	—	—	—	—	—	2
Lysine	57	45	54	8	8	23	54	30	58	57	63	17	24	53	—	17	24	24	24	53
Histidine	12	8	19	Trace	Trace	10	20	13	23	22	28	14	24	11	—	14	24	24	24	11
Arginine	7	11	29	3	5	27	31	16	50	40	106	12	9	30	—	12	9	9	9	30

* Recovered as cysteic acid.

† Recovered as methionine and methionine sulphoxide.

‡ In some cases in which no values are given, the chromatographic peak in the region that ordinarily corresponded to these amino acids was broadened and asymmetric and had a different E_{570}/E_{440} ratio. Further hydrolysis of these samples at 115° for 24 hr. in 6N-HCl did not change these properties.

§ Not acidified before dialysis (separate experiment; see the text).

the EDTA at acid pH, another experiment was carried out in which the EDTA solution removed from the polystyrene boxes was alternately dialysed against neutral water and concentrated until it was free of EDTA, equilibrated for several hours at pH 4.4 at 2° and filtered through a column (3.0 cm. $\times$ 250 cm.) of Sephadex G-25 equilibrated in pyridine-acetic acid buffer, pH 4.4 (Scheme 1).

Analytical procedures. Amino acid analyses were performed on samples hydrolysed in triple-distilled constant-boiling 6N-HCl for 24 hr. at 105°. A commercial model (Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A.) of an automatic amino acid analyser described by Piez & Morris (1960) was used for the amino acid analyses.

The content of hexoses was determined by the anthrone method (Scott & Melvin, 1953), with glucose as a standard.

Ultracentrifugation. Sedimentation coefficients were measured at pH 7.4 in 6M-urea in 0.15M-KCl at 20° in a double-sector cell by using a synthetic boundary in a Spinco model E analytical ultracentrifuge, and the values were corrected to 20° in water ($S_{20,w}$).

Thin-layer chromatography. Weighed portions of material were hydrolysed in 0.1N-HCl for 24 hr. at 100°. A sample was used for the identification of hexoses by thin-layer chromatography on cellulose powder with ethyl acetate-pyridine-water (12:5:4, by vol.) as the solvent (Randerath, 1963). Another sample was further hydrolysed in 4N-HCl at 105° for 5 hr. and used to identify the hexosamines by thin-

layer chromatography as described. The number of μ moles of amino acid/ μ mole of hexose in the samples was determined by amino acid analyses and total anthrone determinations of measured volumes of the samples.

RESULTS

EDTA preparations. Table 1 summarizes the amino acid compositions of the fractions outlined in Scheme 1. Fig. 1 shows a typical elution curve of the EDTA-soluble fraction filtered through Sephadex G-25 resin. The fractions eluted in the region corresponding to proteins or peptides of mol.wt. greater than 3500 and excluded from the column were barely detectable when analysed undiluted at 276m μ . When the material in this region was pooled and dried, it constituted less than 10% of the total sample by weight. Most of the EDTA-soluble material was eluted in the region of small peptides and amino acids. Except for the acid- and EDTA-insoluble fraction, neither 3- nor 4-hydroxyproline was noted in the chromatograms, although the presence of very small amounts would

Fig. 1. Typical elution curve of the EDTA-soluble peptides of erupted bovine enamel filtered through a column (3 cm. $\times$ 170 cm.) of Sephadex G-25 resin in 5% acetic acid, pH 2.5, at 2°. The horizontal bars correspond to the positions of: 1, bovine plasma albumin; 2, amino acids (glycine and aspartic acid); 3, peptides (mol.wt. 250–300); 4, salt.

Fig. 2. Sedimentation-velocity pattern obtained from the non-diffusible water-soluble fraction of the EDTA-soluble portion of adult enamel matrix run in 6M-urea in 0.15M-KCl, pH 7.4, at 20°. The top cell represents a solution at a concentration of approx. 0.4% (w/v) and the bottom cell approx. 0.8% (w/v). The time was 80 min. after reaching the maximum speed of 50780 rev/min. The phase angle is 55°.

not have been detected owing to the low molar extinction coefficient of the hydroxyprolines after reaction with ninhydrin. The relatively high concentration of 4-hydroxyproline and glycine makes it likely that the acid- and EDTA-insoluble residue contained a considerable proportion of collagen. The very high proline content, however, suggests the presence of components similar to those isolated from embryonic bovine enamel (Glimcher *et al.* 1964b).

Because of the extremely high content of phenylalanine in fraction D (EDTA-soluble), and because substances of an aromatic nature may be strongly adsorbed on dextran gels, the tripeptide glycyl-L-phenylalanyl-L-phenylalanine (mol.wt. 369.4) was chromatographed on the Sephadex G-25 resin column. It was greatly retarded, and was eluted in the general region corresponding roughly to that of fraction D.

The major portion of the non-diffusible material that was obtained by dialysis against water, and that was acidified only to pH 4.7 just before Sephadex G-25 filtration, was excluded from the column and was therefore of mol.wt. greater than 3500. One other fraction of lower molecular weight was also collected. The amino acid compositions of these two fractions are also given in Table 1.

The non-diffusible water-soluble fraction (obtained after acidification) was extremely difficult to redissolve after freeze-drying, even when warmed to 35–40° in 6M-urea. Ultracentrifugation (Fig. 2) of that fraction soluble in 6M-urea showed that most of the material was represented by a boundary with a sedimentation coefficient ($S_{20,w}$) of approx. 1.5S. There is a pronounced leading edge to this major boundary and asymmetry of the peak indicating heterogeneity. Two fractions were separated from this material by gel filtration on

Table 2. *Amino acid compositions of the peptides and the free amino acids in EDTA-soluble fraction B*

The numbers at the top of the columns refer to Scheme 2. Values are expressed as residues/1000 total amino acid residues.

	Total amino acid composition (1)	Fractions eluted from Dowex 50 (X8; H ⁺ form) resin column		
		Peptides eluted with water (2)	Eluted with aq. NH ₃	
			Peptides (3)	Free amino acids (4)
Cystine (half)*	< 1	2	—	—
3-Hydroxyproline	—	—	—	—
4-Hydroxyproline	—	—	—	—
Aspartic acid	85	122	117	1
Threonine	31	40	61	21
Serine	64	92	107	30
Glutamic acid	129	203	195	10
Proline	28	18	75	34
Glycine	511	420	188	737
Alanine	58	33	95	144
Valine	27	25	—	—
Methionine†	10	18	13	5
Isoleucine	‡	‡	‡	‡
Leucine	34	‡	‡	‡
Tyrosine	4	3	—	—
Phenylalanine	8	5	19	11
Hydroxylysine	—	—	—	—
Lysine	8	9	72	3
Histidine	Trace	3	27	2
Arginine	3	8	33	1

* Recovered as cysteic acid.

† Recovered as methionine and methionine sulfoxide.

‡ In some cases in which no values are given, the chromatographic peak in the region that ordinarily corresponded to these amino acids was broadened and asymmetric and had a different E_{570}/E_{440} ratio. Further hydrolysis of these samples at 115° for 24 hr. in 6N-HCl did not change these properties.

Table 3. *Amino acid compositions of the hydrochloric acid-soluble components of the organic matrix of erupted bovine enamel*

The capital letters at the top of columns refer to fractions eluted from Sephadex G-25 (Scheme 3 and Fig. 3). Values are expressed as residues/1000 total amino acid residues.

	Fractions eluted from Sephadex G-25 column				
	A	B	C	D	E
Cystine (half)*	4	—	6	—	< 1
3-Hydroxyproline	Trace	—	—	—	—
4-Hydroxyproline	31	—	—	—	—
Aspartic acid	75	104	136	86	67
Threonine	60	47	50	42	32
Serine	74	81	111	117	133
Glutamic acid	104	168	224	176	113
Proline	119	100	26	19	20
Glycine	130	155	283	397	387
Alanine	110	65	65	77	72
Valine	59	46	24	23	29
Methionine†	30	14	1	3	2
Isoleucine	27	28	11	13	17
Leucine	49	63	22	28	31
Tyrosine	5	Trace	3	—	3
Phenylalanine	25	21	5	—	66
Hydroxylysine	27‡	—	3	—	—
Lysine	30	55	20	13	14
Histidine	17	27	8	7	13
Arginine	26	25	1	Trace	Trace

* Recovered as cysteic acid.

† Recovered as methionine and methionine sulfoxide.

‡ This chromatographic peak was more asymmetrical than the standard and may represent other components in addition to hydroxylysine.

Bio-Gel P-30, the major one of mol.wt. greater than 30000 and the smaller fraction of mol.wt. less than 30000. The small amount of material available and its relative insolubility prevented further physicochemical study.

The water-soluble non-diffusible fraction was found to contain approx. 20 μ moles of hexose/ μ mole of amino acid. This corresponds to a carbohydrate content of approx. 95% on a molar or residue basis. The fraction isolated from this material of mol.wt. greater than 30000, from its behaviour on gel filtration, also had a carbohydrate content of approx. 90–95%; the small amount of material of mol.wt. less than 30000 contained approx. 85–88% of carbohydrate. Qualitatively similar results were obtained when the amino acid content was computed on the basis of the recovery of amino acids from known weights of the samples.

Although thin-layer chromatography permitted only qualitative analysis of the carbohydrate components, it was obvious that glucose was by far the most predominant constituent of the hexoses identified in the total non-diffusible fraction, and in the higher-molecular-weight components.

Smaller amounts of galactose, mannose and fucose were also present in these samples. Glucosamine was present in greater amounts than galactosamine in the whole non-diffusible fraction, but galactosamine was the predominant amino sugar in the higher-molecular-weight components. Only galactose could be identified in the lower-molecular-weight fraction, although this may have been due to the small size of the sample used.

The content of peptide-bound and free amino acids in fraction B of the EDTA-soluble material and their compositions (Scheme 2) are shown in Table 2. Except for amino acids with a strong negative charge such as free phosphoserine, free amino acids should be adsorbed on the Dowex 50 (H^+ form) resins at acid pH, and eluted at alkaline pH by aq. ammonia. This was confirmed by chromatographing mixtures of standard amino acids of acid hydrolysates of the enamel proteins on these resin columns. Therefore that portion of fraction B which was eluted with water from the Dowex 50 (H^+ form) resin should have theoretically been composed solely of peptide-bound amino acids. This was confirmed by chromatographing

Fig. 3. Typical elution curve of the HCl-soluble peptides of erupted bovine enamel filtered through a column (3 cm. $\times$ 170 cm.) of Sephadex G-25 resin in 5% acetic acid, pH 2.5, at 2°. The horizontal bars correspond to the positions of: 1, bovine plasma albumin; 2, amino acids (glycine and aspartic acid); 3, peptides (mol.wt. 250–300); 4, salt.

the water-eluted portion directly on the amino acid analyser. Only negligible quantities of free amino acids were identified.

In the fraction eluted from the Dowex 50 (X8; H⁺ form) resin with aq. ammonia, approx. 25% of the amino acids was peptide-bound, the remainder existing as free amino acids. Of the entire amino acid content of fraction B, approx. 80% of the amino acids was peptide-bound.

Hydrochloric acid preparations. Table 3 summarizes the amino acid compositions of the fractions outlined in Scheme 3. Fig. 3 shows a typical elution curve of the hydrochloric acid-soluble fraction filtered through Sephadex G-25 resin. An insufficient amount of the hydrochloric acid-insoluble fraction was recovered for amino acid analysis.

Hydroxyproline was detected only in fraction A. The compositions of peptide-bound and free amino acids in fractions C and D are given in Tables 4 and 5 respectively. Approx. 90% of the amino acids in the aq.-ammonia-eluted portion of both fractions C and D existed as free amino acids, and only 10% or less was peptide-bound. In the entire fraction C, approx. 15% of the amino acids was peptide-bound, and in fraction D 42% of the amino acids was peptide-bound.

DISCUSSION

Many factors combine to make a study of the organic matrix constituents of mature dental enamel difficult. Because of the extremely small amount of protein present in the mature enamel (approx. 0.06% in bovine species) (Glimcher *et al.* 1964c), especially in relation to the adjacent layers of dentine and coronal cementum, which contain approx. 25–30% collagen, very many carefully prepared teeth must be utilized. The use of samples that are either of inadequate size, or not completely free of dentine or cementum, combined with extensive dialysis of material that is largely diffusible, has in the past resulted in either the recovery of insufficient amounts of material for even a single amino acid analysis (Piez, 1962), or the recovery of collagen-like proteins, presumably from the adjacent tissue layers (Hess, Lee & Neidig, 1953; Stack, 1954; Battistone & Burnett, 1956; Rodriguez & Hess, 1963).

The mild demineralization of the enamel of large numbers of teeth in turn requires the use of relatively large volumes of concentrated EDTA. Since most of the matrix constituents are soluble in EDTA and are diffusible, the recovery of small amounts of proteins and peptides from large volumes of concentrated EDTA, by methods that minimize denaturation and peptide-bond hydrolysis, presents many technical problems. To diminish the volume of fluid required, an alternative procedure to the EDTA method was tried. Decalcification of the enamel and solubilization of the organic matrix was carried out by continuously regenerating a fixed volume of fluid into which was dissolved hydrogen chloride gas to maintain the pH at 2.3–2.8. However, despite the low temperature employed (2°), the long time required for demineralization at pH 2.3–2.8 resulted in peptide-bond hydrolysis, as evidenced by the marked increase in the percentage of the material existing as free amino acids compared with the samples demineralized in EDTA. Moreover, in contrast with the method employing EDTA in which contaminating collagen fibrils were not solubilized but recovered in the EDTA-insoluble precipitate, a small amount of collagen was dissolved in the hydrochloric acid and recovered in the fraction excluded from the Sephadex G-25 column together with the higher-molecular-weight components of the enamel matrix. In the procedures employing EDTA at slightly alkaline pH it was difficult to deal with the large volumes of solution directly, either by dialysis or by Sephadex-gel filtration, so that repeated acidification to pH 1.5 was required to precipitate the EDTA and to permit concentration of the solution. To assess the effects of this acidification on the enamel peptides and proteins, a sample

Table 4. *Amino acid compositions of the peptides and free amino acids of hydrochloric acid-soluble fraction C*

The numbers at the top of the columns identify the subfractions as in Scheme 2 for the EDTA-soluble fraction B. Values are expressed as residues/1000 total amino acid residues.

	Fractions eluted from Dowex 50 (X8; H ⁺ form) resin column			
	Total amino acid composition (1)	Peptides eluted with water (2)	Eluted with aq. NH ₃	
			Peptides (3)	Free amino acids (4)
Cystine (half)*	6	—	—	5
3-Hydroxyproline	—	—	—	—
4-Hydroxyproline	—	—	—	—
Aspartic acid	136	65	152	133
Threonine	50	25	—	64
Serine	111	96	91	125
Glutamic acid	224	432	113	237
Proline	26	31	171	10
Glycine	283	232	136	290
Alanine	65	41	82	61
Valine	24	20	46	17
Methionine†	1	10	—	4
Isoleucine	11	7	24	8
Leucine	22	20	65	16
Tyrosine	3	6	19	—
Phenylalanine	5	8	—	3
Hydroxylysine	3	—	Trace	—
Lysine	20	5	52	22
Histidine	8	2	52	6
Arginine	1	1	—	—

* Recovered as cysteic acid.

† Recovered as methionine and methionine sulfoxide.

of the EDTA solution was filtered through Sephadex G-25 without prior acidification. The elution pattern was qualitatively similar to that obtained from EDTA solutions that had been acidified, although the fraction retarded on the Sephadex G-25 column contained fewer free amino acids and somewhat more peptide-bound amino acids: approx. 85–90% of the amino acids was peptide-bound compared with 80% in the samples that were repeatedly acidified. Considering the small sample size used, this small difference could have been due to experimental error. Moreover, the peptides glycyl-L-aspartic acid and glycyl-L-leucylglycine chromatographed on the Dowex 50 (X8; H⁺ form) resin were not hydrolysed into free amino acids. Since the major features that characterized the EDTA-soluble (and acidified) organic constituents were confirmed on samples that were not acidified, and since it is unlikely that peptide bonds were hydrolysed on the Dowex 50 (X8; H⁺ form) resin, it is safe to conclude that the major constituents of the organic matrix are peptides of relatively low molecular weight and are readily diffusible.

The non-diffusible proteins and peptides, including a fraction whose mol.wt. was greater than 30 000 as evidenced by its behaviour in gel filtration, are largely carbohydrate in nature (glycoproteins and glycopeptides) and contain both hexoses and hexosamines. They appear to be somewhat unusual with respect to their relatively high content of glucose. Galactosamine had been previously identified in the organic matrices of embryonic bovine enamel (Glimcher *et al.* 1961).

All the protein and peptide fractions were characterized by their relatively high concentrations of serine. The other amino acids present in significant amounts in the peptides were glycine, glutamic acid and aspartic acid. Glycine was the predominant amino acid present in the small peptides and as the free amino acid.

In several of the serine-rich peptides, partial acid hydrolysis and subsequent chromatography and electrophoresis revealed the presence of phosphoserine, which had previously been identified in the proteins of both embryonic and adult bovine enamel (Glimcher & Krane, 1964). The predominant

Table 5. *Amino acid compositions of the peptides and free amino acids of the hydrochloric acid-soluble fraction D*

The numbers at the top of the columns identify the subfractions as in Scheme 2 for the EDTA-soluble fraction B. Values are expressed as residues/1000 total amino acid residues.

	Fractions eluted from Dowex 50 (X8; H ⁺ form) resin column			
	Total amino acid composition (1)	Peptides eluted with water (2)	Eluted with aq. NH ₃	
			Peptides (3)	Free amino acids (4)
Cystine (half)*	—	—	—	—
3-Hydroxyproline	—	—	—	—
4-Hydroxyproline	—	—	—	—
Aspartic acid	86	139	98	33
Threonine	42	11	—	60
Serine	117	83	29	138
Glutamic acid	176	117	—	216
Proline	19	30	29	8
Glycine	397	440	337	402
Alanine	77	38	73	69
Valine	23	17	39	20
Methionine†	3	4	20	—
Isoleucine	13	13	59	10
Leucine	28	24	170	27
Tyrosine	—	9	93	—
Phenylalanine	—	19	93	—
Hydroxylysine	—	—	—	—
Lysine	13	32	20	10
Histidine	7	21	15	7
Arginine	Trace	4	—	Trace

* Recovered as cysteic acid.

† Recovered as methionine and methionine sulphoxide.

amino acids noted in these peptides and the ones isolated in this study (aspartic acid, glutamic acid and glycine) have also been identified in the phosphate-containing peptides from collagen (Glimcher, Francois, Richards & Krane, 1964a), fibrinogen (Blombäck, Blombäck, & Searle, 1963), ovalbumin (Flavin, 1954); Perlmann, 1952), casein (Mellander, 1963) and calf-intestinal alkaline phosphatase (Engström, 1964). The persistence of serine-containing proteins and peptides in the enamel matrix during maturation, when over 99% of the protein constituents is lost (Glimcher *et al.* 1964b,c), may be due to the strong interaction between the serine-bound organic phosphate and the inorganic crystals (Glimcher & Krane, 1964).

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REFERENCES

- Battistone, G. C. & Burnett, G. W. (1956). *J. dent. Res.* **35**, 260.
 Blombäck, B., Blombäck, M. & Searle, J. (1963). *Biochim. biophys. Acta*, **78**, 563.
 Eastoe, J. E. (1960). *Nature, Lond.*, **187**, 411.
 Eastoe, J. E. (1963). *Arch. oral Biol.* **8**, 663.
 Engström, L. (1964). *Biochim. biophys. Acta*, **92**, 79.
 Flavin, M. (1954). *J. biol. Chem.* **210**, 771.
 Friberg, U. A., Levine, P. T. & Glimcher, M. J. (1964). *Biol. Bull., Wood's Hole*, **126**, 253.
 Glimcher, M. J., Francois, C. J., Richards, L. & Krane, S. M. (1964a). *Biochim. biophys. Acta*, **93**, 585.

- Glimcher, M. J., Friberg, U. A. & Levine, P. T. (1964c). *Biochem. J.* **93**, 202.
- Glimcher, M. J., Friberg, U. A. & Levine, P. T. (1964d). *J. Ultrastruct. Res.* **10**, 76.
- Glimcher, M. J. & Krane, S. M. (1964). *Biochim. biophys. Acta*, **90**, 477.
- Glimcher, M. J., Mechanic, G. L., Bonar, L. C. & Daniel, E. (1961). *J. biol. Chem.* **236**, 3210.
- Glimcher, M. J., Mechanic, G. L. & Friberg, U. A. (1964b). *Biochem. J.* **93**, 198.
- Hess, W. C., Lee, C. Y. & Neidig, B. A. (1953). *J. dent. Res.* **32**, 585.
- Mellander, O. (1963). In *Transfer of Calcium and Strontium across Biological Membranes*, p. 265. Ed. by Wasserman, R. H. New York: Academic Press Inc.
- Perlmann, G. E. (1952). In *Symposium on Phosphorus Metabolism*, vol. 2, p. 167. Ed. by McElroy, W. D. & Glass, B. Baltimore: The Johns Hopkins University Press.
- Piez, K. A. (1961). *Science*, **134**, 841.
- Piez, K. A. (1962). In *Fundamentals of Keratinization*, chapter 11, p. 173. Ed. by Butcher, E. O. & Sognnaes, R. F. Washington, D.C.; American Association for the Advancement of Science (Publication no. 70).
- Piez, K. A. & Morris, L. (1960). *Analyt. Biochem.* **1**, 187.
- Randerath, K. (1963). In *Thin Layer Chromatography*, p. 202. Verlag Chemie; New York and London: Academic Press Inc.
- Rodriguez, M. S. & Hess, W. C. (1963). *Abstr. int. Ass. dent. Res.*, 41st gen. Meet., no. 64, p. 49.
- Scott, T. A., jun. & Melvin, E. H. (1953). *Analyt. Chem.* **25**, 1656.
- Stack, M. V. (1954). *J. Amer. dent. Ass.* **48**, 297.