The Amino Acid Composition of the Organic Matrix and the Neutral-Soluble and Acid-Soluble Components of Embryonic Bovine Enamel

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The proteins of developing dental enamel matrix, although among the least studied of all the extracellular proteins, have nevertheless been found to possess certain unique and interesting properties. The amino acid composition has been characterized by the highest content of proline thus far reported for any protein (approx. 200–250 residues/1000 amino acid residues), a relatively large number of glutamic acid and histidine residues, and the presence of hydroxyproline and hydroxylysine (Eastoe, 1960, 1963; Glimcher, Mechanic, Bonar & Daniel, 1961*a*; Piez, 1961).

Except for collagen and polyproline, the configurations proposed for fibrous proteins cannot accommodate such large amounts of proline (Lindley, 1955; Szent-Györgyi & Cohen, 1957; Glimcher *et al.* 1961*a*). By constructing models a possible configuration was found which permitted as many as 250 residues of proline/1000 amino acid residues to be randomly incorporated into the polypeptide chains (Glimcher *et al.* 1961*a*). The structure proposed was composed of anti-parallel pleated sheets with a 180° bend of the polypeptide chains for each proline residue and was similar to the cross- β configuration suggested by Astbury, Beighton & Parker (1959). Cross- β X-ray-diffraction patterns have been obtained from histologically identified embryonic bovine enamel matrix that had the characteristic amino acid composition (Glimcher, Bonar & Daniel, 1961*b*).

Further characterization of the proteins has been hampered by the extremely limited amount of tissue available and the relative insolubility of the proteins (Eastoe, 1963). In the present study, most of the proteins of the decalcified organic matrix of developing bovine tooth enamel were found to be soluble in cold neutral buffer solution of low ionic strength, and the residue remaining after exhaustive neutral-buffer extraction dissolved in mild acid. The amino acid composition of these fractions was investigated.

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MATERIALS AND METHODS

Preparation of soluble proteins. Unerupted premolar and central incisor teeth were dissected from the lower jaws of 3–7-month-old bovine embryos, and the decalcified cell-free dentine-free enamel matrix was obtained as described by Glimcher *et al.* (1961*a*). In the older embryos, the first permanent molar tooth was also used. The enamel organ was removed within 18–24 hr. and fresh 0.3M-EDTA solution, pH 7.4, was used to obtain the enamel matrix proper. The solubility of the proteins in EDTA was very much less at room temperature than at 2°, and the procedure was therefore carried out at room temperature (approx. 20–22°) in covered trays containing both toluene and merthiolate (1 part per 50 000) for bacteriostasis. Cultures of the enamel matrix at the end of the procedure revealed no bacterial growth.

Immediately after dissection, the decalcified enamel matrix was placed in acetone–water solution (6:1, v/v) at 2°, the EDTA was removed by repeated washing with the acetone–water solution, and samples were dried. This material is referred to as ‘decalcified embryonic enamel matrix’. Other samples of decalcified enamel matrix were prepared by placing the freshly dissected material and the EDTA used in the dissection of the enamel matrix in dialysis tubing, and dialysing against 0.3M-EDTA, pH 7.4, at 2° for 72–96 hr. The EDTA was removed by dialysis against water, and the entire contents of the bags were freeze-dried.

Fractions that were soluble in cold neutral buffer solutions were prepared as follows. Samples of the decalcified enamel matrices were dispersed in 0.3M-EDTA, pH 7.4, at 2°, placed in a dialysis tubing and dialysed against 0.3M-EDTA, pH 7.4, at 2° for 72–96 hr. The EDTA was removed by dialysis against 0.05M-tris buffer, pH 7.4, and the dialysis continued until no further protein dissolved. The suspension was clarified by centrifugation at 2° at 14 000 rev./min. in an International centrifuge for 1 hr., the supernatant was dialysed free of tris against water, and the entire contents of the dialysis tubing were freeze-dried. This fraction is termed the ‘first neutral-soluble fraction’. The residue remaining after the first cold-neutral-buffer extraction was suspended and redispersed in fresh 0.05M-tris buffer, pH 7.4, in stoppered Erlenmeyer flasks and shaken at 2° for approx. 48–72 hr. The supernatant was separated and treated as above and the procedure repeated until only very small amounts of protein or no detectable protein could be extracted from the residue with the neutral tris buffer (‘last neutral-soluble fraction’), as determined by analysis of the extracts for protein content by the method

of Lowry, Rosebrough, Farr & Randall (1951), or by measuring the extinction at 278 μ . The residue was then suspended and dispersed in cold 0.05% acetic acid, the pH adjusted to 3.5 with conc. acetic acid, and the suspension dialysed at 2° against 0.05% acetic acid for 48–96 hr. The acetic acid solution was clarified by centrifugation and freeze-dried. This material is referred to as the 'acid-soluble fraction', and represents the proteins that were insoluble in neutral buffer solutions but soluble in mild acid.

Most of the proteins were precipitated by warming the solutions to 15° or higher, especially if the ionic strength of the solution was greater than 0.1. This was particularly true of the acid-soluble fractions. When the neutral-soluble proteins were acidified to pH 5.0 or less and then dialysed against water, most of the proteins were precipitated. Both the neutral-soluble and acid-soluble proteins were insoluble in salt solutions of ionic strength greater than approx. 0.5–0.6, even at 2°.

Amino acid analysis. Samples of the dried or freeze-dried enamel preparations were hydrolysed in triple-distilled constant-boiling 6N-HCl for 24 hr. at 105°. The amino acid analyses were performed on a commercial model (Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A.) of an automatic amino acid analyser described by Piez & Morris (1960).

RESULTS

About 75–90 % of the proteins of the decalcified organic matrix of developing bovine enamel was soluble in cold neutral buffer solutions of low ionic strength, and the neutral-insoluble residue readily dissolved in dilute acetic acid. The bulk of the neutral-soluble proteins was removed from the decalcified matrices in the first two extractions, although in the experiments reported below at least eight and as many as 15 neutral-buffer extractions were carried out. Over 90 % of the neutral-soluble proteins was dissolved in the first four or five extractions. After the first four or five extractions, decreasingly smaller amounts of protein were dissolved with each successive extraction. Although all of the organic matrices were extracted a minimum of eight times, the extraction was not as complete in the latter as in those instances in which the matrices were extracted 12–15 times. Less than 5 % of the neutral-soluble protein remained in the organic matrices after eight extractions.

Table 1. *Amino acid compositions of decalcified embryonic enamel matrix and its neutral-soluble and acid-soluble components*

Experimental details are given in the text. The results are given as means, $\pm$ S.D. where appropriate.

Amino acid composition (residues/1000 total amino acid residues)

No. of analyses...	Decalcified embryonic enamel matrix (pooled samples)								Decalcified embryonic enamel matrix (single embryo)		
	Incisor 9		Premolar 7		Neutral-soluble fraction				Pre-molar 1		
					First extract		Last extract				
					Acid-soluble fraction						
Incisor 4		Premolar 10		Incisor 4		Premolar 9		Incisor 4		Premolar 13	
Cystine (half)*	1.0 ± 0.60	0.22 ± 0.06	3.0	1.6 ± 1.0	0.4	0.40 ± 0.04	0.5	0.6 ± 0.3	0.2	0.2	
3-Hydroxy-proline†	Trace	Trace	Trace	Trace	0.3	Trace	0.4	0.6 ± 0.1	0.0	0.0	
4-Hydroxy-proline†	Trace	Trace	Trace	Trace	4.0	Trace	5.5	6.2 ± 1.1	0.0	0.0	
Aspartic acid	37 ± 2	33 ± 2	34	32 ± 2	41	38 ± 4	41	42 ± 1	38	34	
Threonine	29 ± 2	27 ± 2	27	29 ± 2	35	34 ± 5	34	33 ± 2	31	29	
Serine	63 ± 6	49 ± 3	47	48 ± 5	74	69 ± 6	91	92 ± 7	69	49	
Glutamic acid	156 ± 6	159 ± 5	161	159 ± 12	144	153 ± 12	144	148 ± 8	160	163	
Proline	213 ± 14	253 ± 17	265	258 ± 4	222	240 ± 17	173	171 ± 13	200	244	
Glycine	70 ± 6	54 ± 4	52	51 ± 3	68	68 ± 8	83	84 ± 3	71	56	
Alanine	22 ± 2	22 ± 2	24	23 ± 1	26	25 ± 4	24	23 ± 2	24	20	
Valine	37 ± 1	38 ± 2	37	38 ± 2	38	39 ± 2	34	32 ± 2	38	39	
Methionine‡	49 ± 4	55 ± 4	44	48 ± 6	36	33 ± 8	47	46 ± 4	45	56	
Isoleucine	30 ± 4	34 ± 2	35	35 ± 3	30	30 ± 4	23	20 ± 2	29	33	
Leucine	96 ± 3	95 ± 2	97	100 ± 5	101	104 ± 3	102	104 ± 6	98	95	
Tyrosine	49 ± 6	47 ± 4	39	42 ± 2	43	39 ± 9	47	46 ± 4	44	49	
Phenylalanine	36 ± 4	26 ± 2	23	23 ± 2	38	30 ± 2	47	50 ± 4	40	26	
Hydroxylysine	2.5 ± 1.0	1.5 ± 0.5	2.7	4.0 ± 1.0	3.0	5.3 ± 1.8	3.5	4.4 ± 1.0	3.3	1.8	
Lysine	19 ± 2	16 ± 1	14	14 ± 1.0	18	15 ± 5	23	23 ± 2	21	16	
Histidine	62 ± 3	73 ± 2	78	79 ± 2	58	62 ± 6	43	41 ± 5	59	72	
Arginine	27 ± 4	19 ± 2	16	15 ± 2	20	19 ± 4	38	37 ± 4	29	18	

* Recovered as cysteic acid.

† See the text.

‡ Recovered as methionine and methionine sulfoxide.

The amino acid compositions of the whole decalcified enamel matrix, of the first and last neutral-soluble fractions, and of the acid-soluble fraction, as well as of the decalcified enamel matrices obtained from the premolar and incisor teeth of one embryo, are given in Table 1. The average values of the last neutral-soluble and the acid-soluble fractions varied somewhat more than those of the decalcified matrices or the first neutral-buffer extracts, since they depended on how extensively the samples had been extracted before the last neutral-buffer extraction.

A small amount of tryptophan (approx. 1 residue/1000 amino acid residues) was identified in all fractions, but because of the very large and variable loss of this amino acid during acid hydrolysis it is omitted from Table 1.

All fractions contained small amounts of 4-hydroxyproline. In most instances, however, the amounts were so small that accurate quantitative analysis was impossible, and therefore in these instances 4-hydroxyproline is simply listed as being present in trace amounts. Only by analysing very large quantities of the proteins of the decalcified enamel matrices, the first neutral-buffer extracts and the last neutral-buffer extract of premolar enamel could 4-hydroxyproline be identified. For example, a hydrolysate of decalcified premolar enamel matrix consisting of a total of about 192 μ -moles of amino acids contained 54 μ -moles of proline, 10 μ -moles of glycine and 0.11 μ -mole of 4-hydroxyproline (approx. 0.6 residue/1000 total amino acid residues). Although the differences were very small, the decalcified enamel matrix and the first neutral-soluble extracts from incisor teeth had slightly higher 4-hydroxyproline contents than did similar fractions from premolar teeth. This difference was even more pronounced in the last neutral-buffer extracts, but no significant difference was noted in the acid-soluble fractions. In addition to 4-hydroxyproline, 3-hydroxyproline (Irreverre, Morita, Robertson & Witkop, 1962; Piez, Eigner & Lewis, 1963) was also identified in each fraction (Fig. 1).

The whole decalcified enamel matrices of premolar teeth had higher proline and histidine contents and smaller serine, glycine and arginine contents than did matrices from incisor teeth. This is also clearly seen in the analyses of the decalcified enamel matrices obtained from the premolar and incisor teeth of one embryo. However, although the compositions of whole decalcified organic matrices of incisor and premolar teeth differed, those of their neutral-soluble and acid-soluble fractions were essentially the same. With successive neutral-buffer extractions, the neutral-soluble fractions of both incisor and premolar enamel showed a slight increase in the serine content and a decrease in the histidine content.

The amino acid composition of the acid-soluble fractions differed in several important respects from the neutral-soluble fractions: the former had higher serine, glycine, phenylalanine and arginine contents and markedly smaller proline and histidine contents.

Fig. 1. Chromatographs obtained on an automatic amino acid analyser of acid hydrolysates of (a) last neutral-buffer extract of premolar bovine enamel, (b) bovine cemental collagen and (c) bovine dentinal collagen, showing the relative amounts of 3-hydroxyproline and 4-hydroxyproline. The 3-hydroxyproline:4-hydroxyproline ratio was about ten times as high in the enamel proteins as in the cemental collagen and three to four times as high as in the dentinal collagen. Peak A, 3-hydroxyproline; peak B, methionine sulfoxide; peak C, 4-hydroxyproline; peak D, aspartic acid.

DISCUSSION

The whole decalcified enamel matrices of incisor teeth of bovine embryos, 3–7 months of age, contained smaller proline and histidine contents and a higher serine content than did similarly aged samples of premolar and molar teeth. That the average amino acid composition of the decalcified enamel matrices obtained from a large number of embryos of different ages really reflects the age, the maturation and development of the tooth, and is not due to a pooling and age-distribution artifact, is evident from the experiments in which the enamel was obtained from the central incisor and premolar teeth of the same embryo. Since the amino acid compositions of the neutral-soluble and acid-soluble fractions of both incisor and premolar teeth were similar it appears that the organic matrices of central incisor teeth contain a smaller proportion of the proline- and histidine-rich components than those of premolar and molar teeth. The central incisor teeth appear earlier, and are developmentally more mature than the premolar or molar teeth (Brown, Christofferson, Massler & Weiss, 1960). This suggests that proline- and histidine-rich and serine-poor components are lost during the development and maturation of the enamel.

Consistent with the suggestion that the maturation of the enamel is accompanied by a selective loss of certain components are the findings of Deakins (1942), who also reported a progressive loss of water and organic materials from enamel with maturation, as well as work by Glimcher, Friberg & Levine (1964a) which has shown a very marked decrease in the protein content of erupted fully developed bovine incisor enamel as compared with embryonic unerupted incisor enamel. Further, there was a considerable difference in the amino acid composition of the enamel proteins of erupted teeth, the most notable features being that they contained smaller proline and histidine contents and higher serine content. These findings are consistent with the hypothesis that there is a progressive loss of the proline- and histidine-rich and serine-poor components with maturation.

Although the neutral-insoluble acid-soluble residue of the organic matrix had smaller proline and histidine contents and higher serine, glycine, phenylalanine and arginine contents, it had many of the characteristic features of the neutral-soluble components. This suggests that the residue contains components whose composition is similar to the neutral-soluble fraction as well as other constituents with a very markedly different composition.

All fractions contained 4-hydroxyproline. In view of the extremely small quantities present and the low molar extinction coefficient of hydroxyproline after reaction with ninhydrin, its reported

absence in human enamel proteins (Eastoe, 1960, 1963) and in water-soluble pig embryonic-enamel proteins (Piez, 1961) is probably due to the small quantities of protein analysed. The slightly increased amounts of 4-hydroxyproline found in incisor enamel as compared with premolar enamel, together with the finding of the presence of a thin layer of calcified collagen (cementum) covering the crowns of both erupted and late unerupted bovine teeth (Glimcher, Friberg & Levine, 1964b), suggests that part of the hydroxyproline may be present as a cemental collagen contaminant that is synthesized relatively late in the development of the tooth. On the other hand, although the amounts were small and difficult to determine, the 3-hydroxyproline:4-hydroxyproline ratio was about ten times as high in the enamel as in the bovine cemental collagen, and about three to four times as high as in the dentinal collagen. Since the two possible sources of a collagen contaminant are the dentine and cementum, this would indicate that 3-hydroxyproline and possibly 4-hydroxyproline are components of either the enamel proteins or of an enamel collagen, rather than components of a dentinal or cemental collagen contaminant. The final resolution of this problem must await the separation and characterization of the individual homogeneous fractions of the neutral-soluble and acid-soluble enamel proteins.

Hydroxylysine was present in all fractions in greater amounts than could be accounted for by contamination with collagen, and therefore must be considered a component of both the neutral-soluble and acid-soluble fractions of the enamel proteins. This confirms previous reports on the decalcified enamel matrix of embryonic bovine teeth (Glimcher *et al.* 1961a) and the water-soluble proteins of embryonic porcine enamel (Piez, 1961). The difficulty in determining hydroxylysine in human enamel (Eastoe, 1960, 1963) is probably due to the small sample size used.

SUMMARY

1. Approx. 75–90 % of the proteins of bovine embryonic enamel have been found to be soluble in cold neutral buffers of low ionic strength, and the residue that was insoluble in neutral buffers was readily dissolved in cold dilute acetic acid. The amino acid compositions of the neutral-soluble fractions differed significantly from that of the acid-soluble fraction.

2. The decalcified organic matrix of incisor enamel had smaller proline and histidine contents and a higher serine content than did the organic matrix of premolar enamel. These differences appear to reflect the earlier development and maturation

of the incisor teeth, and indicate that maturation of enamel is accompanied by a loss of protein components rich in proline and histidine and poor in serine.

3. Small amounts of both 3- and 4-hydroxyproline were detected in all protein fractions. The 3-hydroxyproline:4-hydroxyproline ratio in enamel was higher than that of either bovine dentinal or cemental collagen, suggesting that at least a portion of the hydroxyprolines are true constituents of the organic matrix of developing enamel.

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The Isolation and Amino Acid Composition of the Enamel Proteins of Erupted Bovine Teeth

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Though there has been substantial agreement that the amino acid composition and molecular structure of embryonic enamel proteins are distinctive and distinguish the proteins from collagen (Eastoe, 1960, 1963; Glimcher, Mechanic, Bonar & Daniel, 1961*a*; Piez, 1961; Glimcher, Mechanic & Friberg, 1964*a*; Glimcher, Bonar & Daniel, 1961*b*; Pautard, 1961), the nature of the protein matrix of mature erupted enamel is less clear. A number of investigators have reported the presence of substantial amounts of hydroxyproline in the proteins isolated from mature human enamel (Hess, Lee & Neidig, 1953; Stack, 1954; Battistone & Burnett, 1956; Rodriguez & Hess, 1963). Further, Rod-

riguez & Hess (1963) were able to obtain X-ray-diffraction patterns of the insoluble residue that were characteristic of collagen. However, the amino acid composition of the proteins reported by these investigators, as well as earlier qualitative analyses by Block, Horwitt & Bolling (1949), differed considerably. From the proportions of the basic amino acids histidine, lysine and arginine, Block *et al.* (1949) classified the mature enamel proteins as eukeratins. From a consideration of the X-ray-diffraction patterns obtained from the insoluble matrix of mature enamel, Perdok & Gustafson (1961) also considered the proteins to be modified keratins.

Most of the discrepancies reported appear to be due to the inherent difficulties of obtaining sufficiently large samples of histologically defined

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