The Amino Acid Composition of the Organic Matrix of Decalcified Fetal Bovine Dental Enamel*

MELVIN J. GLIMCHER,† GERALD MECHANIC, LAURENCE C. BONAR, AND EUBERT J. DANIEL

From the Department of Orthopedic Surgery, Harvard Medical School and the Massachusetts General Hospital, Boston 14, Massachusetts

(Received for publication, June 16, 1961)

The organic matrix of dental enamel is produced by morphologically characteristic cells of the enamel organ (ameloblasts), which are derived from the stratified epithelial cells of the primitive oral cavity (1). On the basis of its ectodermal origin, as well as its chemical composition, physical properties, and histochemical staining characteristics, the protein component of the organic matrix, which accounts for the bulk of the organic material, has been classified as a eukeratin (2-6).

Reports of the amino acid composition of the total enamel protein, as well as soluble and insoluble fractions, have varied considerably (2-8). The discrepancies seem to stem primarily from the difficulty of isolating sufficient quantities of enamel protein free from the underlying dentin which contains approximately 40-fold more protein, principally collagen, than fully calcified enamel, and from surface protein "cuticles" (either remnants of the enamel organ or films deposited from saliva).

Previous work from this laboratory has shown that it is possible to separate cell-free ethylenediaminetetraacetate-decalcified enamel matrix from both the cellular enamel organ and the underlying dentinal collagen of fetal unerupted bovine incisor teeth at certain ages of the fetus (9). Furthermore, both the histological structure of the matrix and its molecular configuration as determined by x-ray diffraction are preserved (9). It was thought that an amino acid analysis of the cell-free organic matrix of enamel which had been histologically, histochemically, and crystallographically characterized would prove of value, since this has not been accomplished in the past.

The x-ray diffraction studies previously mentioned (9) revealed that the protein was in the cross-β configuration. Except for the suggested cross-β configuration of actomyosin films after treatment with adenosine triphosphate (10), the enamel protein is the only naturally occurring vertebrate protein thus far reported to be in this particular molecular configuration. The only other naturally occurring cross-β proteins are the silk like protein of the chrysopa egg stalk (11) and the protein of bacterial flagella (12).

Since the nature of the polypeptide chain configuration responsible for the cross-β diffraction pattern is still under investigation (11, 13-15), it was felt that a complete amino acid analysis of a naturally occurring protein which had this particular configuration might provide some help in elucidating the structural problem.

Our further interest in the chemical, as well as the structural, nature of enamel protein stemmed from the increasing evidence that the organic matrices of mineralized tissues are intimately involved in the initiation and control of crystallization (16-19). Dental enamel is the most highly calcified of all vertebrate tissues, and recent electron microscopic studies of the early stages of enamel mineralization (20, 21) have suggested that the organic matrix plays a role similar to that proposed for the collagen of bone and cartilage in the nucleation of the inorganic calcium-phosphate crystals of apatite.

EXPERIMENTAL PROCEDURE

Isolation of Enamel Matrix—Unerupted incisor teeth of the lower jaw of heads from 4- to 6-month-old embryonic Holstein and Angus calves were carefully dissected, wiped clean of surrounding connective tissue and capsular material, and briefly rinsed in cold buffer solution (0.02 M sodium diethylbarbiturate, KCl 1/2 0.165, pH 7.4). A silk suture was placed through the root of each tooth, and the teeth were suspended crown downward in individual vessels containing 0.3 M EDTA, adjusted to pH 7.4. Decalcification was carried out at 4°C, with thymol crystals added for bacteriostasis.

Within 3 to 4 days, two separate soft tissue membranes were seen grossly and were separated from the tooth under the dissecting microscope. The inner one was identified histologically and histochemically as cell-free, microscopically dentin-free enamel matrix proper, and x-ray diffraction patterns of oriented films showed a typical cross-β configuration without any evidence of collagen.

Amino Acid Analysis—The delicate membranes, identified as the cell-free enamel matrix, were carefully washed free of EDTA with cold buffer solution (0.02 M sodium diethylbarbiturate, KCl 1/2 0.165, pH 7.4) over a period of several days, washed free of buffer and salt with distilled demineralized water, placed in previously tared 10 × 75-mm hydrolysis tubes, and dried to constant weight at 105°C in a vacuum oven. Hydrolysis was carried out in triplicate, constantly boiling HCl for 20 hours at 105°C in sealed tubes. The hydrolysates were taken to dryness, washed three times with demineralized distilled water, and pooled to obtain sufficient amounts for quantitative amino acid analysis.

The amino acid analyses were carried out by ion exchange resin...
with the aid of a standard containing 0.05 μM hydroxyproline. Therefore, photographs were taken of the hydroxyproline peaks, magnified approximately three times, and the values computed.

Because of the low extinction coefficient of hydroxyproline when caused to react with ninhydrin, it was very difficult to compute accurately the values from the charts directly. Therefore, photographs were taken of the hydroxyproline peaks, magnified approximately three times, and the values computed with the aid of a standard containing 0.05 μM hydroxyproline.

In addition, aliquots of several of the hydrolysates were analyzed by column chromatography for hydroxyproline by the method of Stegeman (23), and in one sample the eluent of the resin column containing hydroxyproline and aspartic acid was diverted into a 25-ml volumetric flask before mixing with ninhydrin had occurred. The eluent was brought to volume, and an aliquot was analyzed for aspartic acid by the method of Moore and Stein (24). The remainder of the solution was taken to dryness, dissolved in a small amount of water, desalted by the method of Mechanic and Levy (25), and analyzed directly for hydroxyproline by the method of Stegeman (23).

Losses of serine, threonine, hydroxyproline, proline, and tryptophan during acid hydrolysis were taken into account by hydrolyzing a standard mixture of amino acids in exactly the same fashion as was used for the enamel matrix. These corrections were 2 to 4%, except for tryptophan, which was approximately 60%. The magnitude of this latter correction is of little importance because of the small amount of tryptophan recovered.

RESULTS

The average amino acid composition of fetal bovine dental enamel protein expressed as residues per thousand total residues is summarized in Table I. The results are compared with those of developing enamel of human fetal central incisors (7). It should be noted in comparing these values that the latter investigation was carried out on the entire calcified enamel and not the EDTA-decalcified residue and therefore represents both the soluble and insoluble enamel proteins.

The data in Table I show that the amino acid composition of the bovine fetal enamel protein reported here is similar to that reported by Eastoe (7) for human fetal incisor enamel. The characteristic features, already noted by Eastoe (7) are the very high proline content, (higher than for any previously reported protein); a high content of glutamic acid compared to aspartic acid; relatively high methionine content; and tyrosine content exceeding phenylalanine content. The cystine content is very low compared to that of epidermal keratin and other members of the keratin-myosin-epidermin-fibrin class. The most significant difference is our consistent finding of hydroxyproline and hydroxylysine, neither of which was reported to be present by Eastoe (7).

Because of the small number of hydroxyproline residues in the protein and because of the technical difficulties involved in computing the values of the small peaks on column chromatography, the value given in Table I must be considered as only approximate. Direct colorimetric determination of hydroxyproline on aliquots of the hydrolysates or on the hydroxyproline fraction diverted from the resin column gave values of the same order of magnitude, however.

A small peak coming off the column immediately after alanine was noted in all samples of enamel matrix. This was identified as galactosamine in the following manner. By increasing the pH of the buffer (67 ml of pH 2.9 buffer and 8 ml of 0.8 M citrate) the galactosamine peak of standard solution appeared completely separated from the alanine, occurring closer to the valine peak. The small peak of enamel matrix hydrolysates behaved similarly. No glucosamine, which the above modification would separate from glycine, was detected.

DISCUSSION

Although the protein of dental enamel is produced by cells derived from the stratified gingival epithelium, both its molecular configuration (9) and its amino acid composition distinguish it from the characteristic epidermal keratins and collagen (26, 27). The amino acid composition of gingival epithelium was not determined, but x-ray diffraction showed it to be a typical α keratin (9). A recent electron microscopic study of developing enamel (21) has demonstrated that unlike epidermal keratins, which result from the desquamation and degeneration of the squamous epithelial cells, the enamel matrix filaments are formed extracellularly by ameloblasts. Thus, the morphological differentiation of the oral epithelial cells to ameloblasts appears to

* K. A. Piez, personal communication.

### Table 1

Amino acid composition of dental enamel matrix

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Present work</th>
<th>Human fetal enamel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average of 12 analyses</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Glycine</td>
<td>71.5±4</td>
<td>3.3±0.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>20.7±2</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>Valine</td>
<td>34.4±3</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>27.3±2</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>97.2±5</td>
<td>2.1±0.1</td>
</tr>
<tr>
<td>Proline</td>
<td>215.0±11</td>
<td>0.1±0.05</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>3.45±0.07</td>
<td>1.06±0.05</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>26.3±1.6</td>
<td>1.6±0.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>48.8±1.3</td>
<td>2.3±0.1</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.78±0.20</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>Serine</td>
<td>69.6±4.7</td>
<td>4.7±0.2</td>
</tr>
<tr>
<td>Threonine</td>
<td>29.7±1.3</td>
<td>1.3±0.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>48.6±1.0</td>
<td>4.4±0.1</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>1.50±0.04</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td>Hydroxylysine</td>
<td>2.25±0.33</td>
<td>0±0.01</td>
</tr>
<tr>
<td>Lysine</td>
<td>19.3±1.4</td>
<td>1.4±0.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>61.7±3.3</td>
<td>3.3±0.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>26.1±2.6</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>37.4±1.2</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>147.7±5.3</td>
<td>5.3±0.1</td>
</tr>
<tr>
<td>Amide N</td>
<td>175.4±10.5</td>
<td>10.5±0.5</td>
</tr>
</tbody>
</table>

* Corrected for destruction during hydrolysis. † Total of methionine and methionine sulfoxides. ‡ Recovered as cysteic acid.
be accompanied by functional changes both in the method of matrix production and in the configuration and composition of the protein component produced.

A variation in the amino acid composition from sample to sample was found, which was larger than that observed by applying similar techniques to purified proteins; therefore, it is probably not due to experimental error but to variation in the composition of the decalcified enamel matrix. As far as can be determined from the histological, x-ray diffraction (9), and electron microscopic studies, the structure and configuration of the enamel matrix has been preserved in samples prepared as described.

Except for the analyses reported by Eastoe (7) and by Piez and Likins (8), which showed no hydroxyproline, all previous studies of enamel protein have demonstrated the presence of relatively large amounts of hydroxyproline (2-6). The latter appears to be due primarily to contamination with dentinal collagen, since there was also present a high content of glycine and alanine. On the other hand, with the chromatographic techniques and small protein sample (0.8 mg) employed by Eastoe (7), the small amount of hydroxyproline reported here might not have been detected.

Although there appeared to be some correlation between the amount of hydroxyproline found and the total amount of amino acids in any one sample, the inaccuracies in determining the small amounts of hydroxyproline are such that it is not possible to tell whether the hydroxyproline content is proportional to sample weight. Therefore, our results cannot resolve the question of whether hydroxyproline is a constituent of enamel matrix or whether it represents a small (~3%) contamination with collagen.

On the other hand, the presence of hydroxylysine cannot be accounted for on the basis of collagen contamination. Considering that there are 108 residues of hydroxyproline and 13 residues of hydroxylysine per 1000 total residues in bovine dentinal collagen (8), the maximal number of hydroxylysine residues per thousand residues to be expected from collagen contamination based on the hydroxyproline content would amount to approximately 0.4 residue. Since approximately 2.25 residues of hydroxylysine per 1000 residues were found, it can be concluded safely that enamel matrix contains hydroxylysine. Except for collagen and trypsin (28) therefore, enamel protein appears to be the only other protein thus far isolated which contains hydroxylysine.

Whether the galactosamine is part of the enamel protein per se, bound tightly, or a part of a ground substance protein, cannot be answered. Despite extensive investigations, a similar problem has not yet been resolved with respect to the hexosamine content of highly purified collagens. Although no glucosamine was detected, a small amount might have been present, and destroyed during hydrolysis (29).

A number of investigators (30, 31) have indicated that pyrrolidine residues cannot be accommodated in the α-helix. Since approximately one out of every five residues is proline, steric considerations make it unlikely that a significant amount of the protein could be in the α configuration characteristic of the epidermal keratins (32). This has been confirmed by the x-ray diffraction studies previously cited (9).

Preliminary model-building experiments have indicated that it would be difficult to accommodate pyrrolidine residues in the pleated sheet type of structures proposed for the β configurations by Corey and Pauling (32) without considerably disrupting such configurations. It was found, however, that by introducing a 180° bend in the polypeptide chain at each pyrrolidine residue, the pleated sheet structure could be maintained, with the disruption of only one hydrogen bond per pyrrolidine residue. The resulting structure consists of pyrrolidine chains folded back on one another, in a fashion similar to that proposed by Astbury et al. (13), for the cross-β configuration in supercontracted proteins. On the basis of the models constructed, it would appear that the number of residues between adjacent prolines need not be constant; as long as there are two amino acids on the NH₂-terminal end and one on the COOH-terminal end of the amino acid, the hydrogen-bonding pattern can be maintained.

It is significant that model-building experiments have independently indicated that proline can be accommodated in a structure which is similar to that proposed by Astbury et al. (13) for the cross-β configuration, and that the x-ray diffraction studies of enamel protein do in fact show the cross-β pattern (9).

If the organic matrices of mineralized tissues are intimately concerned with the mechanism of crystallization by providing sites for the heterogeneous nucleation of the inorganic crystals (34), it is of interest that a protein for which both molecular structure and amino acid composition differ from collagen is also able to initiate the formation of apatite crystals.

### SUMMARY

The histologically and structurally characterized enamel protein matrix of 4- to 6-month-old fetal bovine incisor teeth, which is insoluble in ethylenediaminetetraacetic acid, neutral buffers, and water, has been isolated and its amino acid composition determined. Its amino acid composition readily distinguishes it from epidermal keratins and collagen. It is characterized by a very high proline content (215 residues per 1000 residues) and the presence of hydroxylysine. Although a small amount of hydroxyproline was found in all samples, it is not possible to say whether this represents contamination with collagen, or whether the enamel protein contains hydroxyproline.

Model-building experiments which have taken into account the high proline content have independently indicated that existence of a cross-β structure is compatible with the previously reported x-ray diffraction results.

### Acknowledgments

The authors are indebted to Dr. Philip T. Levine of Tufts University School of Dental Medicine for valuable suggestions and collaboration in preparing the dental enamel, and to Dr. Paul Goldhaber of the Harvard School of Dental Medicine for helpful discussion during the course of this work.

We also gratefully acknowledge the assistance of Mr. Jack Snider of Snider Brothers, Inc., meat packing house, and Charles Gilvarg, D.V.M., United States Department of Agriculture, for their cooperation in securing and determining the ages of the embryos used.

---

1 Unpublished results from this laboratory.
2 Piez and Likins have reported in a private communication that they have since recovered small amounts of hydroxyproline in embryonic porcine enamel and that their more recent analyses are substantially similar to those reported here.

---
REFERENCES

1. ORBAN, B. J., in Oral histology and embryology, Ed. 4, C. V. Mosby Company, 1957, Chapter II, p. 34.
2. BLOCK, R. J., HORKWITZ, M. K., and BOLLING, D., J. Dental Research, 28, 618 (1949).
5. BATTSONE, G. C., and BURNETT, G. W., J. Dental Research, 35, 260 (1956).
The Amino Acid Composition of the Organic Matrix of Decalcified Fetal Bovine Dental Enamel
Melvin J. Glimcher, Gerald Mechanic, Laurence C. Bonar and Eubert J. Daniel