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.

This study was supported by research grants from the U.S. Public Health Service, National Institutes of Health (DE 01777, AM 06375-02), and the John A. Hartford Foundation Inc., to the Harvard Medical School and the Massachusetts General Hospital. The authors thank Miss Mary R. Gonzalves for her help in the dissection of the enamel, Miss Alice Scholtz for technical assistance in the preparation of the proteins, and Mrs Daulet Ramamoorthy and Mr Yuri Ripninsky for their help with the amino acid analyses. We also acknowledge the assistance of Mr Jack Snider of Snider Brothers Inc. meat packing house, and Charles Gilvarg, D.V.M., U.S. Department of Agriculture, for their co-operation in securing and determining the ages of the embryos used.

REFERENCES

Astbury, W. T., Beighton, E. & Parker, K. D. (1959). *Biochim. biophys. Acta*, **35**, 17.
Brown, W. A. B., Christofferson, P. V., Massler, M. & Weiss, M. G. (1960). *Amer. J. vet. Res.* **21**, 7.
Deakins, M. (1942). *J. dent. Res.* **21**, 429.
Eastoe, J. E. (1960). *Nature, Lond.*, **187**, 411.
Eastoe, J. E. (1963). *Arch. oral Biol.* **8**, 663.
Glimcher, M. J., Bonar, L. C. & Daniel, E. J. (1961*b*). *J. molec. Biol.* **3**, 541.
Glimcher, M. J., Friberg, U. A. & Levine, P. T. (1964*a*). *J. Ultrastruct. Res.* **10**, 76.
Glimcher, M. J., Friberg, U. A. & Levine, P. T. (1964*b*). *Biochem. J.* **93**, 202.
Glimcher, M. J., Mechanic, G., Bonar, L. C. & Daniel, E. J. (1961*a*). *J. biol. Chem.* **236**, 3210.
Irreverre, F., Morita, K., Robertson, A. V. & Witkop, B. (1962). *Biochem. biophys. Res. Commun.* **8**, 453.
Lindley, H. (1955). *Biochim. biophys. Acta*, **18**, 194.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Piez, K. A. (1961). *Science*, **134**, 841.
Piez, K. A., Eigner, E. A. & Lewis, M. S. (1963). *Biochemistry*, **2**, 58.
Piez, K. A. & Morris, L. (1960). *Analyt. Biochem.* **1**, 187.
Szent-Györgyi, A. G. & Cohen, C. (1957). *Science*, **126**, 697.

Biochem. J. (1964), **93**, 202

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

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(*Received* 10 *January* 1964)

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, Rodriguez & 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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enamel for analysis. Because of its extremely low protein content (approx. 0·5 %) (Losee & Hess, 1949; Stack, 1954; Battistone & Burnett, 1956; Burnett & Zenewitz, 1958), attempts to isolate mature enamel proteins free of contamination by the contiguous layer of dentinal collagen have proved difficult. The two tissues interdigitate at the dentino-enamel junction, and the bovine dentine used in the present study, containing about 25 % of collagen, contains approx. 300–500-fold more protein than the mature bovine enamel, which was found to contain a total of about 0·06 % of protein. The inclusion of even minute amounts of dentine in an enamel sample would therefore result in a significant contamination of the enamel proteins with collagen. For example, the proteins isolated from a sample of bovine enamel contaminated with 1 % of dentine would contain about 70–80 % of dentinal collagen. With bovine teeth there is another potential source of collagen contamination. The surface of erupted bovine enamel is covered with a layer of calcified collagen which is continuous with the cementum (Glimcher, Friberg & Levine, 1964*b*). In addition to collagen contaminants, the surface of enamel may be covered with extraneous proteins deposited from the saliva and from the desquamated squamous epithelium lining the oral cavity.

Repeated attempts at isolating the decalcified organic matrix of enamel from fully erupted bovine incisor teeth by decalcification in EDTA, as described for unerupted embryonic bovine teeth (Glimcher *et al.* 1961*b*), or by decalcification in various organic and inorganic acids, failed. The organic matrix of enamel from erupted teeth, unlike that of embryonic enamel, was found to be almost completely soluble in EDTA and in acid media as well.

In order to characterize the enamel proteins, it was therefore necessary to develop methods for obtaining 'pure' enamel. Variations of two basic techniques were used. In the first, fully calcified enamel was dissected free of dentine and cementum under direct microscopic observation. In the second method, relatively impervious materials were applied to all but one area of the central portion of the labial surface of the crown of the tooth after the coronal cementum had been removed by decalcification in EDTA, and the enamel proteins were obtained by taking advantage of their relative solubility in EDTA in comparison with the relative insolubility of the dentinal and cemental collagens in EDTA.

EXPERIMENTAL

Tissue source. The four central permanent erupted incisor teeth of 18–36-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 calcified enamel. The crowns of the teeth were placed in 0·5 M-EDTA, pH 8·3, at 2° or 25° for 24–48 hr. Toluene was added for bacteriostasis. At the end of this time, a thin membrane that covered the entire crown was removed under the dissecting microscope, and was identified as a coronal layer of cementum (Glimcher *et al.* 1964*b*). The exposed surface of the enamel was gently brushed in EDTA, and the crowns were rinsed in double-distilled demineralized water and then dried at 25° over P_2O_5 in a vacuum desiccator. The crowns were then cut into 400 μ thick cross-sections on a modified Gillings–Hamco apparatus (Hamco Machine Co. Inc., Rochester, N.Y., U.S.A.). The middle one-third of the enamel from the labial surface of the crown was removed from the individual cross-sections of the teeth in the following fashion. Under the dissecting microscope, a pointed scalpel blade was used to inscribe a line on the enamel parallel to the dentino-enamel junction. This line was placed sufficiently away from the junction on the enamel side, so that a definite layer of enamel remained after breaking or chipping off small pieces of enamel at the inscribed line. This method was extremely tedious and time-consuming, but provided samples of microscopically pure enamel tissue for analysis. A modification of this method was also used, in which a commercial N_2-driven abrasive cutting unit was employed for the dissection of the enamel. The equipment was modified so that the entire dissection could be carried out under direct microscopic observation and the motions of the cutting nozzle in relation to the fixed cross-section of the crown remotely controlled (Friberg, Levine & Glimcher, 1964). In this modification, the cementum was not removed from the crown by decalcification in EDTA, but both it and the outer one-third of the enamel were dissected from 1600 μ thick cross-sections of untreated crowns. The middle one-third of the enamel was obtained for analysis by further dissection of the cross-sections at the dentino-enamel junction. The isolated slabs of calcified enamel were carefully examined under the dissecting microscope by two observers and an enamel slab was discarded when it appeared to be contaminated with either dentine or cementum.

Because of the varying thickness of the coronal cementum and because the width of the abrasive path precluded leaving a visible strip of enamel at the dentino-enamel junction, it was not possible to be certain that microscopically pure enamel was obtained by using the air–abrasive method of dissection.

Recovery of protein from samples of calcified enamel. The enamel proteins were obtained by several different procedures. (1) The enamel was decalcified and the proteins were hydrolysed in 6 N-HCl at 105° for 12–16 hr. The solution was cooled to 25° and calcium phosphate crystals were precipitated by adjusting to pH 8·5–9·0 with NaOH. The solution was clarified by centrifugation and the calcium phosphate precipitate was thoroughly washed with water adjusted to pH 9·0 with NaOH, and the washings were added to the solution. 1·0 M-Sodium phosphate solution, pH 9·0, was added, resulting in further precipitation, and the procedure repeated several times. The washed calcium phosphate precipitates were hydrolysed in 6 N-HCl at 105°

for 24 hr., the HCl was removed by evaporation, and the precipitates were redissolved and analysed for ninhydrin-positive compounds by the method of Moore & Stein (1954). By using this method as a criterion, no adsorbed protein was detected in the washed calcium phosphate precipitates. The protein solution was then dried and hydrolysed for an additional 12–16 hr. in 6N-HCl at 105°. The amino acid composition of such preparations represents the total protein, peptide and amino acid content of the enamel, and such preparations are referred to as 'whole enamel'. Other samples of 'whole enamel' were prepared for analysis by desalting acid hydrolysates (6N-HCl at 105° for 24 hr.) on columns of Dowex 50 (X2; H^+ form) resin (Mechanic & Levy, 1959). (2) Samples of enamel were decalcified in 0·5M-EDTA, pH 8·0, at 2° and the solutions exhaustively dialysed against water. In some experiments, the entire contents of the dialysis bags were analysed (total non-diffusible proteins). In others, the solutions were centrifuged after decalcification in EDTA (EDTA-soluble proteins), and after dialysis against water (water-soluble proteins). Any precipitates that were recovered (EDTA-insoluble proteins, water-insoluble proteins) were thoroughly washed and analysed separately.

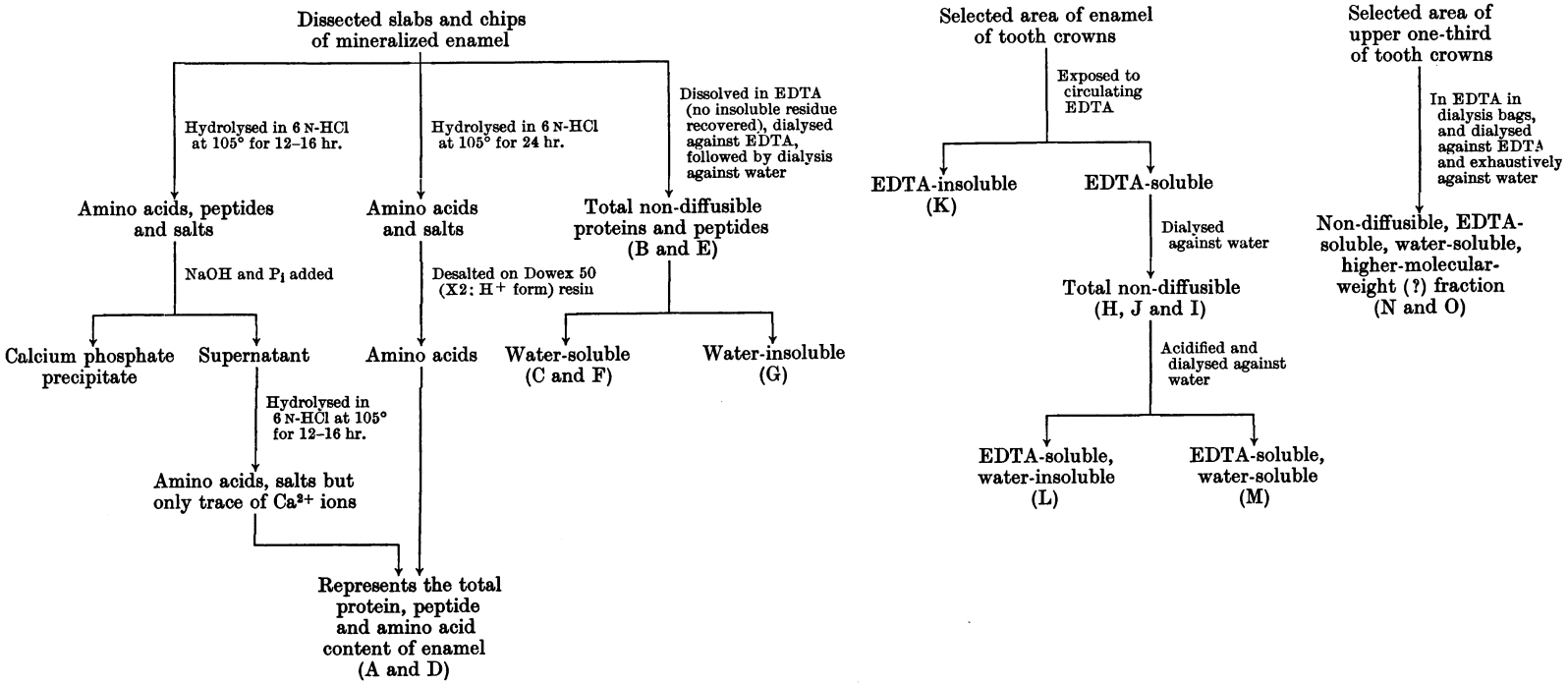
Isolation of enamel proteins by decalcification of selected areas of the labial surface of the tooth crown. The rationale of this procedure was as follows. After removal of the outer layer of coronal cementum in EDTA as described above, the pulp chamber, the lingual surface and all but a 1·0 cm. × 1·0 cm. area of the central portion of the labial surface of the enamel was covered with relatively water-impervious material. Decalcification of the exposed area in EDTA, terminated while a layer of undecalcified enamel still remained covering the dentine, ensured that the source of both the soluble and insoluble material recovered originated only from the enamel. The central portion of the labial surface of the crown was used since the enamel of the incisal region and the lingual surface were much thinner than the central labial enamel, and decalcification of the whole crown would have exposed the dentine underlying the enamel of the incisal region and lingual surface before the enamel of the central portion of the labial surface was decalcified. Two methods were used to carry out this procedure. (1) The crowns of teeth from which the cementum had been removed in EDTA were dried at 25° *in vacuo* over P_2O_5 and placed lingual surface down in small polystyrene boxes filled with liquid dental acrylic resin and catalyst. The tooth was then positioned with a glass rod so that the labial surface partially protruded and was level. The acrylic resin was allowed to harden for 15–30 min., and more acrylic resin added so as to cover all but a 1·0 cm. × 1·0 cm. area of the central portion of the labial surface. The resin was then allowed to harden for an additional 72–96 hr. (2) The pulp chambers of the sectioned crowns were filled with the dental acrylic resin, small polystyrene rods were inserted, and the acrylic resin was allowed to harden. By using the polystyrene rod as a handle to manipulate the tooth crown, the surface of the crown was coated with a commercial brand of (finger)nail varnish (Cutex; Chesebrough–Ponds Inc., New York, N.Y., U.S.A.), leaving a 1·0 cm. × 1·0 cm. area of the central labial portion unpainted. After the first layer had dried, one or more additional coats of nail varnish were applied, and the polystyrene rods cut at the pulpal opening.

Tooth crowns prepared by both methods were placed in covered polystyrene trays containing 0·5M-EDTA, pH 8·0–8·5, at 8–10°. The EDTA solution was circulated continuously by means of a peristaltic pump, and changed at 24–72 hr. intervals. In some experiments the EDTA solutions from the various time-periods were analysed separately, and in others all of the EDTA solutions were pooled and analysed as one sample. The teeth were carefully observed during the procedure, and coronal and cross-sections made of randomly selected samples to insure that an adequate thickness of undecalcified enamel still remained covering the dentine over the exposed labial surface. Several experiments were carried out with the upper one-third of the crown where the cemental layer is very thin. After removal of the cemental collagen in EDTA, the surface of the crowns, which were somewhat softened, were abraded with a sharp blade while submerged in EDTA, rinsed in distilled water, left in EDTA solution for 24 hr., washed and dried. All but a small area of the labial surface was covered with dental acrylic resin, and the tooth crowns were dialysed against EDTA for approx. 72–120 hr. at 2°. The contents of the dialysis bags were clarified and the supernatants dialysed for 10–16 days against 0·05M-tris buffer, pH 7·4, containing KCl (0·15M), at 2°, followed by dialysis against water, and the solutions clarified and freeze-dried. Toluene was used both in the dialysis bags and the dialysing media. Culture of the contents of the dialysis bags at the end of the procedure revealed no bacterial growth.

The EDTA- and water-insoluble and -soluble proteins were recovered from the EDTA solutions as described above or, because of the large volumes of EDTA used, by the following procedure. After clarification of the EDTA solutions by centrifugation, most of the EDTA was precipitated by adjusting the pH to 1·5 at 2°. The supernatant was separated from the precipitate by filtration through a medium sintered-glass filter, the precipitate was washed on the filter with water adjusted to pH 1·5, and the filtered washings were added to the original filtrate. The filtrate was then neutralized and concentrated on a rotary evaporator, the pH adjusted to 1·5, and the precipitated EDTA again removed by filtration. This procedure was repeated several times and the solution finally desalted either by dialysis against water or on a column of Sephadex G-25. The procedures used are summarized in Scheme 1.

Amino acid analyses. Except for the samples partially hydrolysed and dissolved with the mineral intact as described above, samples used for amino acid analyses were 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.

Hydroxyproline determination. In addition to the resin-column chromatography utilizing the automatic amino acid analyser, hydroxyproline was also determined as follows. Hydroxyproline was separated from known volumes of the acid hydrolysates used for amino acid analyses by chromatography on a column (0·9 cm. × 10·0 cm.) of Dowex 50 (X12; H^+ form), resin (13–23 μ spherical particles) equilibrated and eluted with 2N-HCl (Stern, Mechanic, Glimcher & Goldhaber, 1963). The hydroxyproline in the fraction eluted from the column was determined colorimetrically (Stegemann, 1958) with smaller sample and reagent volumes, which permitted the detection of as little as 1·0 μg.



Scheme 1. Procedures employed in obtaining the proteins of erupted bovine enamel. The capital letters in parentheses refer to the columns in Table 1.

RESULTS

From the recovery of amino acids in hydrolysates of fully mineralized samples that were not dialysed or desalted on Sephadex G-25, the total protein, peptide and amino acid content of erupted bovine incisor teeth was approx. 0·06 %. From 50 to 90 % of the proteins, peptides and amino acids was lost, however, either on dialysis or by desalting the EDTA solution on columns of Sephadex G-25.

Table 1 summarizes the results of the amino acid analyses of the various fractions isolated. By using the 4-hydroxyproline content as a measure of the degree of contamination with either dentinal or cemental collagen, it is clear that only by first removing the outer layer of coronal cementum, and then dissecting chips of enamel from relatively thin cross-sections of the crown by hand, a method that permits one to leave a visible layer of enamel at the dentino-enamel junction, was it possible to get samples of calcified enamel essentially free of dentinal or cemental collagen (columns A and D in Table 1). When both the cementum and the enamel were dissected by the abrasive cutting technique, the varying thickness of the coronal cementum and the inability to leave a visible layer of enamel at the dentino-enamel junction apparently resulted in the inclusion of a very small amount of collagen in the enamel preparations.

Although 50–90 % of the proteins was lost on dialysis, the amino acid compositions of the total protein content of enamel and of the non-diffusible fractions were not remarkably different. Neither 3- nor 4-hydroxyproline is noted as occurring in either the fully mineralized samples or in the non-diffusible fractions obtained by the hand microdissection technique. However, trace amounts were occasionally noted by colorimetric analysis of fractions eluted from the Dowex 50 (X12; H^+ form) resin column. In those samples in which hydroxyproline was detected, it amounted to less than 2 residues/1000 amino acid residues. The failure to observe hydroxyproline on the chromatograms obtained from the automatic amino acid analyser in these samples is probably the result of the small amounts present and the low molar extinction coefficient of the hydroxyprolines after reaction with ninhydrin reagent. In those instances where the values of 4-hydroxyproline and hydroxylysine/1000 amino acid residues are listed as approximate numbers, only small absolute amounts, difficult to calculate accurately, were detected on the chromatograms.

Almost all of the proteins were soluble in EDTA, whether obtained from dissected calcified chips or slabs of enamel, or by the exposure of selected areas of the labial surface of the crown of the tooth to EDTA. In only one instance, in which a very large number of teeth were used, was an EDTA-insoluble residue obtained (column K, in Table 1). In the enamel slabs prepared by abrasive dissection, the collagen (or hydroxyproline-rich fraction) was obviously insoluble in EDTA (columns D, E, F and G in Table 1), but because of the small quantity present was not recovered on centrifugation of the relatively large quantity of EDTA used for demineralization.

The EDTA-soluble proteins were also almost completely soluble in water, and except in one instance, where large amounts of enamel were demineralized, insufficient water-insoluble material was obtained for analysis. However, because it had been previously found that the proline-rich neutral-soluble proteins of developing bovine enamel were precipitated on dialysis against water after they had been acidified (Glimcher *et al.* 1964*a*), the EDTA-soluble proteins obtained from 2500 incisor teeth were acidified to pH 2·0, and then dialysed against water at 2°. The solution remained clear after acidification, but, on subsequent dialysis against water, a small amount of a flocculent precipitate was recovered, and the amino acid composition of this fraction (column L in Table 1) was found to be very similar to that of the decalcified organic matrix and the neutral-soluble fraction of embryonic bovine, porcine and human enamel (Eastoe, 1960, 1963; Piez, 1961; Glimcher *et al.* 1961*a*, 1964*a*).

Although the amino acid compositions of the different preparations varied, indicating that each of the samples was undoubtedly a mixture of several distinct species, there were, with few exceptions, certain characteristics common to all of the samples regardless of the method of preparation: relatively high serine, glutamic acid and glycine contents, and in many fractions a relatively high aspartic acid content. In samples prepared by dissolving selected areas of the crown, marked differences in the glycine and aspartic acid contents were noted in various preparations (columns H, I and J in Table 1), but all contained relatively high serine contents.

In those experiments that utilized the relatively deeply situated enamel from selected areas of only the upper one-third of the crowns, and in which the crowns were decalcified in dialysis bags and the EDTA was removed by exhaustive dialysis, the amino acid composition of the non-diffusible and presumably higher-molecular-weight fractions of the water-soluble proteins showed a marked increase in the serine and aspartic acid contents (columns N and O in Table 1). In one of these samples, which contained a total of approximately 35 μmoles of amino acids, a small amount of 4-hydroxyproline was detected.

On several occasions an incompletely resolved peak was eluted slightly earlier than cysteic acid during amino acid analysis. Although in the

Table 1. *Amino acid composition of erupted bovine enamel proteins*

Experimental details are given in the text. Columns A, B, C, D, F, N and O represent averages of two analyses. All other columns represent single analyses. (*a*) Total protein, peptide and amino acid content of the enamel; (*b*) fraction of the total enamel proteins and peptides that was non-diffusible or not retarded on Sephadex G-25; the proteins and peptides obtained from calcified enamel slabs or chips (columns B, C, E, F and G) were samples of the same preparations used for total protein, peptide and amino acid content (columns A and D); (*c*) fraction of the total non-diffusible proteins and peptides that was soluble in water; (*d*) fraction of the total non-diffusible proteins and peptides that was insoluble in water; (*e*) and (*f*) separate experiments; (*g*) water-soluble and -insoluble after acidification (see the text).

	Amino acid composition (residues/1000 total amino acid residues)													
	Calcified enamel							Enamel proteins obtained by decalcification of selected areas of the crowns in EDTA						
	Microdissected by hand			Microdissected by abrasive technique				Total non-diffusible (*b*)			Extracted for 96 hr.			
		Non-diffusible			Non-diffusible									
	Whole enamel (*a*)	Total (*b*)	Water-soluble (*c*)	Whole enamel (*a*)	Total (*a*)	Water-soluble (*c*)	Water-insoluble (*d*)	First 36 hr. (*e*)	72–108 hr. (*e*)	48–96 hr. (*f*)	EDTA-insoluble, diffusible and non-diffusible	Non-diffusible, EDTA-soluble, water-insoluble (*g*)	Non-diffusible, EDTA-soluble, water-soluble (*g*)	Upper one-third of crowns in dialysis bags; non-diffusible, water-soluble*
	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)	(M)	(N) (O)
Cystine (half)†	11	11	14	2	7	9	4	22	23	8	11	3	41	8 22
3-Hydroxyproline*	0	0	0	Trace	0	0	0	0	0	0	Trace	0	0	0 0
4-Hydroxyproline*	0	0	0	~ 10	~ 15	Trace	0	6	0	0	12	Trace	0	~ 2 0
Aspartic acid	94	88	84	49	70	76	61	100	92	66	118	55	76	193 220
Threonine	48	53	56	47	55	57	52	60	64	54	45	30	55	43 40
Serine	102	108	146	109	146	154	195	120	110	132	123	77	136	157 220
Glutamic acid	128	134	140	141	136	153	138	134	146	83	114	153	138	109 88
Proline	90	80	88	121	77	82	73	65	95	108	67	210	101	55 41
Glycine	195	161	154	182	159	150	167	157	119	236	149	70	138	80 76
Alanine	59	73	76	71	92	82	92	82	85	87	91	26	94	56 46
Valine	36	45	37	32	33	35	32	43	41	31	48	37	35	43 40
Methionine‡	5	~ 1	9	18	17	16	13	5	10	41	~ 1	42	Trace	9 3
Isoleucine	28	31	25	23	26	26	24	25	26	23	29	30	30	23 24
Leucine	67	71	54	63	60	58	49	52	58	46	68	92	61	54 49
Tyrosine	7	13	7	17	6	4	3	13	12	Trace	Trace	40	3	16 11
Phenylalanine	39	40	32	25	26	29	19	27	37	17	32	31	13	25 17
Hydroxylysine	~ 1	~ 1	~ 1	~ 1	~ 1	~ 1	0	Trace	~ 1	0	~ 1	~ 1	0	~ 1 ~ 1
Lysine	35	42	34	39	27	27	28	38	40	36	46	20	29	50 54
Histidine	26	16	21	31	19	18	30	26	21	27	16	60	19	46 19
Arginine	29	33	24	19	28	22	19	24	20	33	31	23	30	30 29

* See the text.
† Recovered as cysteic acid.
‡ Recovered as methionine and methionine sulphoxide.

particular chromatographic system employed serine phosphate is not ordinarily separated from cysteic acid, the presence of a prominent acidic peak, coupled with the high concentrations of serine found in the protein fractions, suggested that some of the serine was present in the protein as its phosphorylated derivative, and was not completely hydrolysed to serine even after strong acid hydrolysis. Therefore a small portion of one of the samples that showed the double peak in the region of cysteic acid was subjected to high-voltage paper electrophoresis. A ninhydrin-positive spot was obtained that moved identically with authentic serine phosphate at pH 1·9. However, because of the limited amount of protein available, it was not possible to characterize this component. Further, because in most instances serine phosphate cannot be distinguished from cysteic acid either by elution times or the extinction of the ninhydrin-treated amino acids at either 570 or 440 mμ with the chromatographic system employed in the present study, the values of half-cystine noted in Table 1 may be the sum of cysteic acid and serine phosphate.

DISCUSSION

Previous reports on the amino acid composition of the organic matrix of enamel from erupted teeth have consistently shown the presence of large amounts of hydroxyproline and other amino acids found in collagen (Hess *et al.* 1953; Stack, 1954; Battistone & Burnett, 1956; Rodriguez & Hess, 1963). Dr S. M. Weidmann, Leeds University, has kindly sent us the results of recent unpublished amino acid analyses of carefully prepared human mature enamel. These have shown only 14 residues of hydroxyproline/1000 amino acid residues in the water-soluble and EDTA-soluble fractions, but 54 residues of hydroxyproline/1000 amino acid residues in the insoluble fraction (Lofthouse, 1961). In the early phases of the present study, similar results were obtained in samples of enamel prepared by hand microdissection of cross-sections of tooth crowns obtained from the outer one-quarter to one-third of the labial enamel, leaving very wide strips of enamel behind at the dentino-enamel junction so as to ensure the exclusion of dentinal collagen. However, the surfaces of the crowns were treated only by mechanical brushing and cleaning. The finding of a collagen-like protein in these samples, in which no dentine was included, led to a morphological study of the enamel surface. It was found that the crowns of erupted bovine teeth were covered with a layer of calcified collagen, which was continuous with the cementum covering the roots of the teeth (Glimcher *et al.* 1964*b*). When this layer was removed from the crowns by decalcification and dissection in EDTA before microdissection of the enamel, no detectable hydroxyproline was found on amino acid analysis of the entire mineralized samples, and the amino acid composition was markedly different from that of collagen (column A in Table 1). Although the cemental collagen was grossly removed by decalcification in EDTA, the proteins obtained by decalcification of the outer layers of selected regions of the labial surface of the crowns invariably contained hydroxyproline (column H in Table 1), whereas those obtained from the deeper layers of enamel by subsequent exposure to EDTA contained no detectable hydroxyproline (columns I and J in Table 1). The hydrolysates of the deeper portions of the enamel were approximately three times as large as the samples representing proteins removed during the first 36 hr. of exposure to EDTA, so that failure to identify hydroxyproline was not the result of analysing insufficient amounts of protein. The results indicate that the cemental collagen fibrils penetrate relatively deeply into the enamel.

Although the present study was carried out on bovine erupted teeth, whereas previous studies (Hess *et al.* 1953; Stack, 1954; Battistone & Burnett, 1956; Rodriguez & Hess, 1963) were carried out on human teeth, a recent investigation of carefully dissected human enamel has shown an outer surface layer that histochemically stained like collagen (Rodriguez & Hess, 1963). On solubilization, material was recovered which had the X-ray-diffraction characteristics of collagen, and which contained large amounts of hydroxyproline, glycine, proline and alanine in concentrations similar to those found in collagen. It would therefore appear that the samples of human erupted enamel that have been reported to contain large amounts of hydroxyproline, and whose amino acid composition closely paralleled that of collagen, were contaminated with significant amounts of dentinal collagen, or possibly collagen from the surface of the enamel. Since most of the proteins of mature enamel are diffusible, even a trace of a non-diffusible collagen contaminant in the preparation, before dialysis, would constitute a major proportion of the proteins after prolonged dialysis.

Although small quantities of hydroxyproline were found in many of the water-soluble fractions, it is impossible to say whether hydroxyproline is a constituent of the mature enamel proteins, or whether it represents a trace contamination of the enamel proteins with collagen. A similar problem in interpretation has arisen with embryonic enamel proteins (Glimcher *et al.* 1961*a*), although in the latter recent findings (Glimcher *et al.* 1964*a*) have demonstrated that the 3-hydroxyproline:4-hydroxyproline ratio is much greater in the embryonic enamel proteins than in either dentinal or cemental collagen. This indicates that 3-

hydroxyproline and possibly 4-hydroxyproline are constituents of embryonic enamel proteins. With the proteins of mature enamel, insufficient amounts of protein were available to assess this ratio, even though trace amounts of 3-hydroxyproline were noted in several samples.

From the hydroxyproline content, many samples contained more hydroxylysine than could possibly be accounted for by a collagen contaminant, and it therefore seems likely that hydroxylysine is a component of erupted enamel proteins as well as embryonic enamel proteins (Piez, 1961; Glimcher *et al.* 1961*a*, 1964*a*).

The amino acid composition of the entire organic matrix of erupted enamel is not only distinctly different from that of collagen, but also from that of embryonic bovine, porcine and human enamel matrix (Eastoe, 1960, 1963; Piez, 1961; Glimcher *et al.* 1961*a*, 1964*a*). Further, there is also a remarkable difference in the total amount of protein present. Eastoe (1960) has reported that the protein content of unerupted embryonic human teeth is approx. 20 %. Preliminary results on bovine teeth have shown values as high as 30 %. Since the protein content of the enamel of erupted bovine incisor teeth was found to be approx. 0·06 %, it is obvious that more than 99 % of the enamel proteins are lost during the development and maturation of the enamel, at a time when the tissue is heavily impregnated with inorganic crystals. The loss of organic matrix and water from enamel with increasing calcification and maturation has been reported by Deakins (1942).

Recent studies have shown that about 75–90 % of the proteins of the decalcified organic matrix of developing bovine enamel of embryos 3–7 months of age is soluble in cold neutral buffer solutions, and that the neutral-soluble fraction is characterized by its high proline, histidine and glutamic acid contents (Glimcher *et al.* 1964*a*). After exhaustive neutral-buffer extractions, the insoluble residue contained a markedly decreased proline content and an increased serine content. Thus, in addition to the major fractions of embryonic enamel proteins which are rich in proline and histidine, there is also a small amount of protein(s) with a relatively high serine content. In the present study of mature erupted enamel proteins, just the opposite situation was found: the proline- and histidine-rich components constituted only a small proportion of the total proteins present, the major protein components being characterized by their relatively high serine contents. Therefore the very marked difference in the amino acid composition of the whole enamel matrix of embryonic teeth as compared with mature adult erupted teeth appears to be due to the relative amounts of the various protein components present, and reflects, primarily, the loss of proline- and histidine-rich protein fractions during tooth development. That this loss of protein matrix during maturation, particularly of the proline- and histidine-rich components, begins before eruption of the tooth comes from previous studies in which it was found that developmentally older embryonic unerupted central incisor enamel contained proportionately smaller quantities of the proline- and histidine-rich components than the developmentally younger premolar and molar teeth.

Unpublished studies of the neutral-soluble embryonic enamel proteins have shown that a major component (rich in proline and histidine) is a high-molecular-weight non-diffusible aggregate. Since most of the mature enamel proteins are diffusible and are retarded on columns of Sephadex G-25, their molecular weights must be less than 3500. It is tempting to speculate, therefore, that the progressive loss of protein during the development and maturation of the enamel is accompanied and possibly preceded by a depolymerization of the proteins.

SUMMARY

1. Several methods are described for the isolation of the proteins of enamel from erupted mature bovine incisor teeth. The amino acid composition of the proteins was characterized by the presence of relatively high serine, glutamic acid and glycine contents. Several fractions contained considerable amounts of aspartic acid.

2. Although the amino acid composition of the entire organic matrix differed markedly from that of collagen and from embryonic bovine enamel, a fraction was isolated whose amino acid composition was similar to that of the proline- and histidine-rich neutral-soluble fraction of the embryonic enamel proteins. The relative amount of this component was markedly decreased in the enamel of mature erupted teeth as compared with embryonic teeth, indicating that most of the enamel proteins that are lost during development and maturation are components rich in proline and histidine.

This study was supported by research grants from the U.S. Public Health Service, National Institutes of Health (DE–01777, AM 06375–02), and the John A. Hartford Foundation Inc., to the Harvard Medical School and the Massachusetts General Hospital, and from the Colgate–Palmolive Co. and the American Chicle Co. to the Forsyth Dental Center, Division of Advanced Study and Dental Research. The authors thank Miss Mary Gonzalves and Mr John Brady for their help in dissection of the enamel, and Dr Anthony Andrikides, Mrs Dora Kossiva, Miss Catherine Rutstein and Miss Diane Brickley for their help in the preparation of the protein samples. We are also grateful to Mrs Daulet Ramamoorthy and Mr Yuri Ripninsky for their help with the amino acid analyses.

REFERENCES

Battistone, G. C. & Burnett, G. W. (1956). *J. dent. Res.* **35**, 260.

Block, R. J., Horwitt, M. K. & Bolling, D. (1949). *J. dent. Res.* **28**, 518.

Burnett, G. W. & Zenewitz, J. A. (1958). *J. dent. Res.* **37**, 590.

Deakins, M. (1942). *J. dent. Res.* **21**, 429.

Eastoe, J. E. (1960). *Nature, Lond.*, **187**, 411.

Eastoe, J. E. (1963). *Arch. oral Biol.* **8**, 663.

Friberg, U. A., Levine, P. T. & Glimcher, M. J. (1964). *Biol. Bull., Wood's Hole*, **126**, 253.

Glimcher, M. J., Bonar, L. C. & Daniel, E. (1961*b*). *J. molec. Biol.* **3**, 541.

Glimcher, M. J., Friberg, U. A. & Levine, P. T. (1964*b*). *J. Ultrastruct. Res.* **10**, 76.

Glimcher, M. J., Mechanic, G. L., Bonar, L. C. & Daniel, E. (1961*a*). *J. biol. Chem.* **236**, 3210.

Glimcher, M. J., Mechanic, G. L. & Friberg, U. A. (1964*a*). *Biochem. J.* **93**, 198.

Hess, W. C., Lee, C. Y. & Neidig, B. A. (1953). *J. dent. Res.* **32**, 585.

Lofthouse, R. W. (1961). M.Sc. Thesis: University of Leeds.

Losee, F. L. & Hess, W. C. (1949). *J. dent. Res.* **28**, 512.

Mechanic, G. L. & Levy, M. (1959). *J. Amer. chem. Soc.* **81**, 1889.

Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.

Pautard, F. G. D. (1961). *Arch. oral Biol.* **3**, 217.

Perdok, W. G. & Gustafson, G. (1961). *Arch. oral Biol.* **4** (suppl.), 70.

Piez, K. A. (1961). *Science*, **134**, 841.

Piez, K. A. & Morris, L. (1960). *Analyt. Biochem.* **1**, 187.

Rodriguez, M. S. & Hess, W. C. (1963). *Abstr. int. Ass. dent. Res.*, 41*st gen. Meet.*, no. 64, p. 49.

Stack, M. V. (1954). *J. Amer. dent. Ass.* **48**, 297.

Stegemann, H. (1958). *Hoppe-Seyl. Z.* **311**, 41.

Stern, B. D., Mechanic, G. L., Glimcher, M. J. & Goldhaber, P. (1963). *Biochem. biophys. Res. Commun.* **13**, 137.

Biochem. J. (1964), **93**, 210

The Isolation of D-Fucosamine (2-Amino-2,6-dideoxy-D-galactose) from Polysaccharides of *Bacillus*

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(*Received* 14 *February* 1964)

Until 1954, D-glucosamine was the only amino sugar known to occur in bacteria. More than ten amino sugars have been isolated from bacteria since then (Sharon, 1964). Most bacterial strains appear to contain only two compounds of this class, namely glucosamine and muramic acid. A limited number of bacterial species is, however, capable of producing a larger variety of amino sugars. These include species of the genus *Bacillus*, which, in addition to glucosamine and muramic acid, form galactosamine (Sharon, 1957), a diamino sugar (Sharon & Jeanloz, 1960) and D-fucosamine.

The present paper describes the isolation and identification of D-fucosamine (2-amino-2,6-dideoxy-D-galactose) from polysaccharides produced by two organisms, *Bacillus licheniformis* and *Bacillus subtilis*. This amino sugar was found for the first time in a specific lipopolysaccharide of the Gram-negative *Chromobacterium violaceum* (NCTC 7917) (Crumpton & Davies, 1958). It was subsequently isolated from *B. licheniformis* by Sharon, Shif & Zehavi (1962). [*B. licheniformis* was previously known as *B. subtilis* (ATCC 9945). In the last edition of the *American Type Culture Collection, Catalogue of Cultures* (1958), it has been designated as *Bacillus licheniformis* (ATCC 9945). Indeed, tests in our Laboratory have shown that this organism exhibits scant growth in glucose broth under anaerobic conditions, a property characteristic of *B. licheniformis* (Breed, Murray & Smith, 1957). The organism obtained from Dr Baddiley (Armstrong, Baddiley & Buchanan, 1960) did, however, grow well under the same conditions, and is properly called *B. subtilis*.] A brief report on the occurrence of fucosamine in an unidentified *Bacillus* species and in *Bacillus cereus* has appeared (Leatherwood, Rollins, Kulkarni & Wheat, 1963), and L-fucosamine has been found in the *Pneumococcus* type V polysaccharide (Williams, 1960; Barker, Brimacombe, How, Stacey & Williams, 1961).

A preliminary account of this work has been given (Sharon *et al.* 1962). The final proof of the structure of D-fucosamine, by its synthesis from D-galactosamine, has been described in a preliminary note (Zehavi & Sharon, 1963).