Further Tests on Human Enamel Protein

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In previous work (Pincus, 1936, 1939) it was shown that human enamel protein differs from the keratins amongst which it had been classed (Rosebury, 1930). It contained 12.1% N, 1.2% S, while cystine was either absent or present in low concentration; tyrosine was also present. Enamel protein resisted digestion both by pepsin and trypsin, and was unaffected by KCN, Na_2S , and sodium thiolacetate. This limited examination has now been extended.

METHODS

Preparation of material. Unruptured human molar teeth were used for experiments throughout; these buried teeth were removed surgically from patients aged from 18 years upwards, and kept at room temperature in water saturated with chloroform. The growth of moulds and bacteria has been avoided in the present experiments by using the protein within a few days after extraction of teeth.

suggested that the contents of grooves on the occlusal (biting) surface of molar teeth sometimes exceeded the mere surface layer known as Nasmyth's membrane (Nasmyth, 1839). This appearance was thought to be due to debris of section cutting. Table 1 shows results at this stage of the work. However, the existence of two parts in enamel protein has now been recognized, and separate examination of them has been carried out. After the exposure of the teeth to acid (as above), the protein sometimes falls away from the enamel, lying loose. When this occurs, Nasmyth's membrane is seen as a diaphanous structure, while the groove protein is more nearly a tiny solid mass, sometimes of yellowish tinge. Separation was carried out visually under water by the use of a smooth-ended glass pipette 6–8 cm. long, and about 7 mm. diameter. The original name, Nasmyth's membrane, has been retained for the surface layer, i.e. the protein adjacent to the enamel surface; the other part is termed groove protein. Fig. 1 shows the relation of these structures to each other and to the tooth.

Fig. 1a.

Fig. 1b.

Fig. 1 (a). Diagram of molar tooth. Position of occlusal groove is marked by a circle. (b) Diagrammatic enlargement of circle in (a); the positions occupied by Nasmyth's membrane and groove protein in the occlusal groove are indicated.

The teeth were brushed freely with 0.5% Na_2CO_3 solution and with water, the roots separated from the crowns and discarded. Protein was separated from enamel by steeping in 0.5M-HCl for 24 hr. at room temperature. Dentine was then discarded, and the tiny collections of protein washed in water. Tests have all been carried out on specimens about 3 or 4 mm. diameter at their largest and probably less than 0.1 mm. thick. Prior to 1939, microscopic examination had

RESULTS

Three N estimations shown in Table 1, carried out on mixed Nasmyth's membrane and groove protein, average 11.8%. The low N and the presence of carbohydrate (see Table 2), provide some evidence as to the biochemical nature of these structures; in

considering the estimations, difficulties met in separating tooth proteins from Ca salts must be borne in mind. The N estimations may be compared with those given by Levene (1925) for mucoproteins, where values range from 6.7% in oviduct (Giacosa, 1882) to 13.3% in submaxillary glands (Hammarsten, 1888).

Table 1. *Analysis of Nasmyth's membrane mixed with groove protein*

(a) Quantitative tests (as %, corrected for ash)		
	(Schoeller)	(Weiler)
N	12.1	11.1, 12.1
S	1.2	—
(b) Qualitative tests (hydrolysis at 108°, 30 min., pH 8.0)		
Xanthoproteic	+	
Biuret	- or faint	

Table 2 shows that differences of a biochemical nature exist between the two proteins in occlusal grooves. Cupriethylenediamine (CuEn) stains Nasmyth's membrane black, and there is some reason to believe that a Cu protein complex is formed. Some CuEn protein complexes have been examined, and have been found to be bacteriostatic and possibly bactericidal. The groove protein dissolves immediately in CuEn. The purple colour seen on acid hydrolysis of groove protein is absent when Nasmyth's membrane is subjected to acid hydrolysis; the Molisch test is positive with groove protein and negative with Nasmyth's membrane. Cupriammonium solutions dissolve groove protein immediately while Nasmyth's membrane resists for

days and is stained black. Since the Molisch test shows the presence of carbohydrate, the possible origin of this carbohydrate is of interest. Glycogen was shown by Glock (1940) to be very abundant in mouth epithelium and in other structures adjacent to the developing molar teeth of foetal and very young rats. Examination for the presence of pentose (in the present work) proved negative. Symington & Rankin (1908) have shown X-ray pictures in which human molar teeth may be seen calcifying downwards from the tip of each cusp, the sides eventually meeting to form a groove. Coalescence of cusps to form a complete occlusal surface occurs normally, and in such a process, developmental material may be 'trapped' in the groove.

While relatively few suitable teeth are available, a rigid selection of material ensures that films of mucin or other adventitious material are kept at a minimum. It is obvious that the tenuous layer on the surface of newly formed enamel, Nasmyth's membrane, is soon removed from exposed surfaces by wear, while in secluded positions such as narrow fissures on the occlusal surface, it may persist. This persistence may also apply to such other contents of fissures as are described above, and may lend to the present work an added value. Such narrow fissures are a very common site of dental caries.

Table 2 shows that exposure to Schweitzer's solution results in histological differences in the structure of groove protein becoming apparent. One part disappears promptly; this is thought to be the remnant 'caught up' as it were during the formation of the tooth, in the groove between the cusps, and

Table 2. *Qualitative tests on Nasmyth's membrane and groove protein separately*

	Nasmyth's membrane	Groove protein
Conc. HCl at 37° for 24 hr.	Dissolves	Dissolves; purple colour
Conc. HCl; boiling water bath	Dissolves > 30 min.	Dissolves < 2 min.; purple colour
Millon	+	+
Ninhydrin	-	Not tested
Pepsin at 37°	Very slow attack	Very slow attack
Trypsin at 37°	Very slow attack	Very slow attack
Gram - bacillus at 37° (see note a)	No attack seen in 14 days	Not tested
<i>Lactobacillus acidophilus</i> at 37°	Not tested	No attack seen in 14 days
Schweitzer's solution at room temperature	Resists	The greater part dissolves; a small part resists (see note b)
Cupriethylenediamine at room temperature	Resists for days and is stained black	Dissolves in few seconds
Thioglycolic acid at room temperature	Resists 24 hr.	Dissolves in about 1 min.
Acetic acid	Swells	Not tested
Formol (10%)	Toughened	Toughened
Molisch	-	+
Aniline acetate	-	-
Phloroglucinol	Not tested	Red colour; ppt. No pentose detected spectroscopically

(a) This Gram - bacillus was isolated from dental carious lesions (Pincus, 1948), and its proteolytic action on some proteins (e.g. serum, sericin) at 37° has been studied. The rise in free amino groups which is noted during the first week or so of digestion is sometimes followed by a decrease, due to subsequent bacterial degradation. Estimations were thus made over the period where the titre of free amino groups was rising, i.e. where the proteolytic power of the organism could be demonstrated.

(b) Microscopical examination showed markings resembling enamel prisms, with a few epithelial cells.

persisting there. This remnant is of mesodermal origin. That part of groove protein, which resists exposure to Schweitzer's solution, is probably a fragment of Nasmyth's membrane which existed in histological continuity with the groove protein, and was inadequately separated from it by the use of a pipette, and the gentle suction used.

In its resistance to chemical and bacterial attack, and to pepsin and trypsin, some resemblance between Nasmyth's membrane and silk fibroin had been pointed out to the writer by the late Sir Patrick Laidlaw prior to 1939. Later, the work of Takamatsu (1933) came to notice, describing the solution of silk in cupriethylenediamine, after which a test of the effect of this substance on enamel proteins seemed to be of interest. Clinical trial of local application of cupriammonium solutions for caries prevention is contemplated, when a suitable technique has been evolved.

SUMMARY

1. Human dental enamel protein has been shown to consist of two proteins, Nasmyth's membrane and groove protein; results of some tests on these are given.

2. Both contain tyrosine, and both resist enzymic attack. Groove protein contains carbohydrate, possibly glucose. In cupriammonium solutions the two structures are sharply differentiated, Nasmyth's membrane resisting solution and staining black, while groove protein readily dissolves.

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Properties of Glucose Oxidase (Notatin)

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A glucose oxidase was discovered by Müller (1928, 1936) in *Aspergillus niger* and *Penicillium glaucum*. He found that this enzyme catalyzes the oxidation of glucose to gluconic acid by means of molecular oxygen, and that at a much slower rate it also oxidizes mannose and galactose but not other sugars. Since the activity of this enzyme was found by him not to be affected by cyanide he concluded that it does not depend upon the presence of an active heavy metal.

The enzyme was reinvestigated by Franke & Lorenz (1937) and by Franke & Deffner (1939) who, working with a more purified preparation, demonstrated that during the catalytic oxidation of glucose to gluconic acid, O_2 is reduced to H_2O_2 . They found also a certain proportionality between the activity of different preparations and their flavine content and concluded that the enzyme must be a flavo-protein. Although their enzyme preparation was 80 times more active than that of Müller, we now realize

that it was not more than 10% pure. Unlike Müller they found that in addition to O_2 other substances such as indophenols may also act as hydrogen acceptors although their efficiency is much lower.

Müller (1941), from a study of the oxidation of various sugars by his enzyme preparations both in O_2 and anaerobically using 2:6-dichlorophenolindophenol, concluded that two enzymes were present, one reacting directly with O_2 and a second which reacts only with certain other H acceptors. Franke (1944) has criticized this conclusion of Müller and also those of Ogura (1939) who claimed to have isolated from *Aspergillus oryzae* a glucose-oxidizing enzyme which could not utilize O_2 as H acceptor. According to Franke (1944) the glucose oxidases from *Penicillium glaucum*, *Aspergillus niger* and *A. oryzae* are virtually identical and consist of a single aerobic oxidase. The findings of Müller (1941) and Ogura (1939) may have resulted from the low purity of their preparations; their Q_{10} values being of the