Analytical and ultrastructural studies of pellicle on primary teeth

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The pellicle on permanent enamel has been thoroughly studied. The aims of this study were to compare the chemical composition, rate of formation, and ultrastructural appearance of pellicle formed on deciduous enamel in children with those on permanent teeth. This was done by amino acid analyses, Auger analyses, and transmission electron microscopy. The amino acid composition of 2-h pellicle on deciduous and permanent enamel had an overall similar pattern, but the contents of serine, glycine, and tyrosine were statistically significantly different. An initially slower pellicle formation and a thinner 2-h pellicle without a globular structured second layer was observed on deciduous enamel. The results indicated therefore distinct differences in chemical composition, rate of formation, and ultrastructural appearance between pellicle on primary teeth and that on permanent teeth. □ *Amino acid analyses; Auger analyses; deciduous enamel; protein adsorption; transmission electron microscopy*

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The formation of dental plaque implies the initial adsorption of oral bacteria to the acquired enamel pellicle. It has been shown that the pellicle formed on various dental filling materials and dental enamel varies in amino acid composition and that early plaque formed on these materials varies both quantitatively and qualitatively (1). It has never been studied whether initial microbial colonization differs between various types of dental hard tissues (2). The composition of the initial microflora may vary on various dental hard tissues, such as deciduous and permanent enamel. A first step to evaluate this is to compare the amino acid composition of the acquired enamel pellicles on deciduous and permanent enamel. Solids with similar chemical compositions but with different surface charges have been shown to have a marked influence on the type of proteins they adsorb, as shown by amino acid analyses of the adsorbed integuments (3). Human dental enamel, deciduous and permanent, is known to be of overall similar chemical composition, although studies by Naujoks et al. (4) and Cutress (5) indicate variations in several minor components, including carbonate. Carbonate substitutions in the hydroxyapatite crystals are known to change the surface charge and the solubility of the enamel surface (6–8). This may lead to variations in the chemical composition of the pellicle, and hence to variations in the initial bacterial adsorption.

The acquired enamel pellicle on permanent enamel in adults has been thoroughly studied, whereas studies on the amino acid composition and rate of formation of the acquired enamel pellicle on deciduous enamel have not been reported. It would therefore be of interest to compare the chemical composition, rate of formation, and ultrastructural appearance of pellicle formed on deciduous enamel in children with pellicle on permanent teeth.

In the present study we used Auger electron spectroscopy and depth profiling to study the rate of formation of pellicle on deciduous enamel in situ, transmission electron microscopy to study the appearance of pellicle on deciduous enamel in situ, and amino acid analyses to study the amino acid composition of pellicle formed in vivo on deciduous teeth. These data on pellicle formation on deciduous enamel were compared with the present knowledge of pellicle on permanent teeth in adults (9, 10).

Materials and methods

Pellicle collection and amino acid analyses

Five children with mixed dentitions, age 9–10 years, volunteered for the study. Pellicle material from deciduous teeth was collected from the deciduous molars and, if present, the deciduous canines. Pellicle material from the permanent teeth was collected from the permanent incisors and the permanent first molars. After the buccal surfaces of the teeth had been pumiced and rinsed with water, pellicle was allowed to form for 2 h, during which the study subjects refrained from eating and drinking. Before collection of the 2-h pellicle, the surfaces were rinsed with water, air-dried, and isolated with cotton wool rolls. The pellicle material was collected on a polyvinylidene difluoride (PVDF) membrane by scraping the coronal two-thirds of the buccal surfaces with an enamel hatchet (Black 83, Ash,

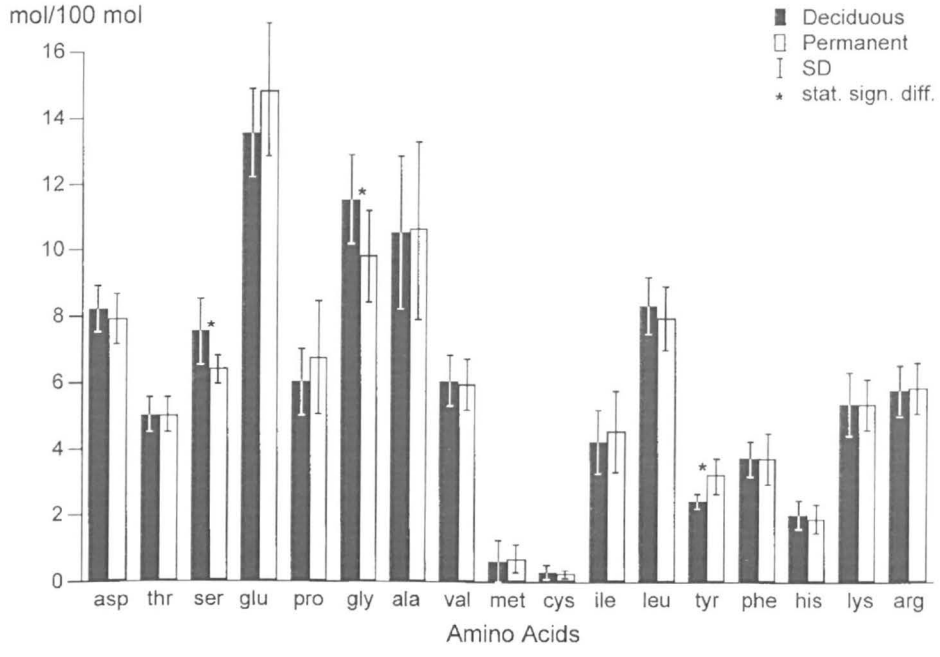

Fig. 1. Amino acid profiles of pellicle material collected in vivo after 2 h; mean of three samples from each individual from deciduous and permanent teeth.

England) and wiping off the collected material on the PVDF membrane. The samples were frozen until analyzed. Pellicle material was collected in three sittings from each individual and was taken from the primary and the permanent teeth in the same sitting. The pellicle material collected from each type of tooth and from each sitting was analyzed separately. The amino acid analyses were performed on an Amino Acid Analyzer (Applied Biosystems, model 421) connected to an Apple Macintosh computer.

Auger electron spectroscopic analyses

To study the rate of formation of pellicle on deciduous teeth, cleaned enamel pieces were worn by three children, age 8–11 years, in situ for 5, 10, 15, 20, 30, 45, 50, 70, and 120 min. Two of the children also participated in the pellicle collection for the amino acid analyses.

Immediately after removal from the mouth, the enamel pieces were rinsed gently in water before they were air-dried. The adsorbed protein film was subjected to Auger electron spectroscopy (AES) and depth profiling by argon ion sputtering as described by Skjørland et al. (10). The sequential sputtering time was 1/20th min, using an argon ion beam with 3 kV energy and 100 $\mu A/cm^2$ ion current density. The detection of nitrogen and oxygen on the enamel pieces was taken as an indication of the presence of organic material. A sudden change to low levels of nitrogen concomitant with a sudden increase in calcium was taken as the beginning of the enamel surface.

Transmission electron microscopy

To study the ultrastructural appearance of the salivary pellicle at various formation times, pumiced deciduous enamel pieces were carried in the buccal sulcus of the mouth by one child (age 11 years) for 30 sec and 1, 15, 30, 60, 90, and 120 min. The child participating in this part of the study also participated in the other two parts of the study. After removal from the mouth, the enamel pieces were gently rinsed in a phosphate buffer solution (pH 7.4) and fixed for 2 h in 2.5% glutaraldehyde. Postfixation was performed in 2% osmium tetroxide for 2 h. Specimens were then dehydrated in an ascending series of ethanol and embedded in Araldite M. Decalcification of the samples was done in 4% ethylenediaminetetraacetic acid (EDTA), pH 7.2, after which the samples were re-embedded. Ultrathin sections from all specimens were cut on an ultramicrotome (ultracut E, Reichert, Germany) equipped with a diamond knife (Microstar 55°, Mikrotechnik, Germany). Serial ultrathin sections were mounted on Piloform-F-coated copper grids (Wacker-Chemie, Germany) and contrasted with uranyl acetate and lead citrate. Examinations were performed in a transmission electron microscope (TEM 201,

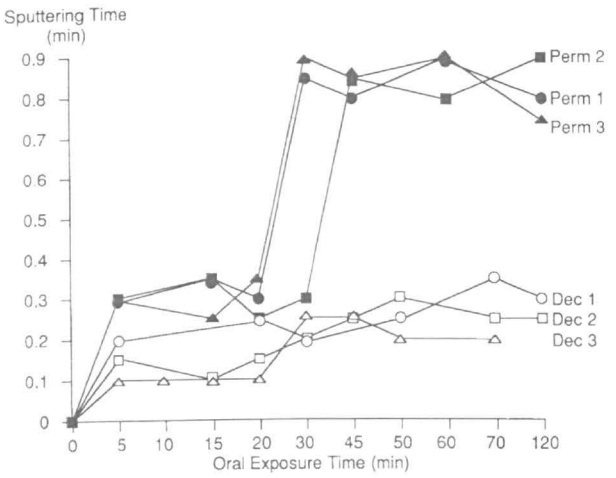

Fig. 2. Rate of pellicle formation on deciduous enamel (Dec) worn by three children and compared with the pellicle formation rate on permanent enamel (Perm) worn by three adults. The pellicle formation rate is presented as a function of oral exposure time and time necessary to remove the integument by argon ion sputtering. Sputtering time indicating pellicle thickness on the ordinate.

Philips, Netherlands) at 80 kV. Representative micrographs were obtained at a magnification of ×30,000.

Statistical method

The data from the amino acid analyses were statistically evaluated using Minitab statistical software. The paired *t* test was used to evaluate differences in amino acid composition between pellicle material from deciduous and permanent enamel.

Results

The comparison of the mean amino acid compositions of 2-h pellicle on deciduous and permanent teeth is shown in Fig. 1.

Pellicle collected from deciduous teeth showed significantly more serine ($P < 0.05$) and glycine ($P < 0.02$) than the 2-h pellicle collected from permanent teeth. The pellicle from permanent teeth also had significantly more tyrosine ($P < 0.02$) than the pellicle from deciduous teeth.

The pellicle formation on the deciduous enamel samples showed a slow rate and is presented together with previously published results for permanent teeth (10) in Fig. 2. The amounts of protein increased from 5 min and seemed to reach a steady level after about 30 to 45 min. The rate of pellicle formation of the three subjects tested in this study followed the same general pattern, with only small variations.

The TEM pictures of pellicle on deciduous enamel at various times are shown in Fig. 3. In all pictures a relatively dense protein layer can be observed. The protein layer increased somewhat in thickness with time, showing relatively homogeneous structures throughout the pellicle thickness.

Discussion

Several studies have contributed to the present knowledge of the amino acid composition, rate of formation, and ultrastructural appearance of the acquired pellicle. All studies, however, have been of pellicle on permanent enamel in adults (9–14).

In this study a comparison of the amino acid composition of pellicle collected from deciduous enamel with that of pellicle collected from permanent enamel in the same mouth was done, using statistical evaluation. An overall similar pattern of the relative amounts of the amino acids could be seen, but the amino acids serine, glycine, and tyrosine were present in statistically significantly different amounts in the two types of pellicle investigated. Statistical evaluation of the amounts of amino acids in various pellicle materials has been used in an earlier study (15), although no conclusions were attempted because four individuals were considered to be insufficient for statistical evaluation. In the present study variations in the amount of serine may be caused by disintegration during acid hydrolysis of the pellicle sample, although the same time of hydrolysis was used for all pellicle samples and therefore should result in a comparable degradation of serine. Acid hydrolysis will also disintegrate tryptophan, cystine/cysteine, and methionine to various degrees (16, 17). The hydrolysis will not cause disintegration of tyrosine and glycine, and disintegration therefore cannot explain the variations found in this study. The results of the statistical evaluation may therefore present a difference in protein composition of the pellicles. Statistical evaluation may result in not showing significant differences, as large variations caused by method and apparatus are added to the biologic variations. The number of individuals in this study limits the conclusion. A generalizing conclusion would need a larger study group. In other studies comparisons of amino acid compositions of pellicle are done by visually comparing graphs and profiles (11, 13). Smaller differences are difficult to detect by this method.

The differences in amino acid composition indicate that there may be different amounts of some proteins or that there are different proteins in the acquired pellicle on deciduous enamel compared with that on permanent enamel. This may be a result of variations in the surface charge (3) and may eventually lead to variations in the initial microflora that adsorbs to the pellicle (1).

In our study and in a similar study (10) in which Auger analyses were used to evaluate the thickness of

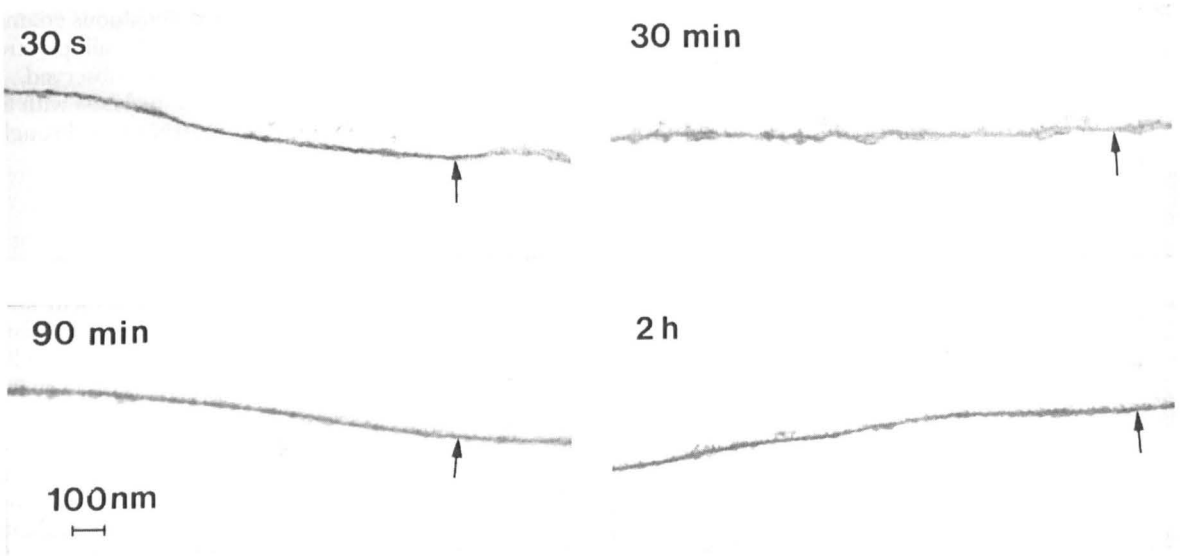

Fig. 3. Transmission electron microscopic pictures showing the ultrastructural appearance of acquired pellicles formed on deciduous enamel in situ after a) 30 sec, b) 30 min, c) 90 min, and d) 2 h. Arrows indicate pellicle towards the enamel. (Magnification, ×30,000.)

the acquired pellicle, some limitations of the method should be borne in mind. The density of the material will, at least to some extent, influence the sputtering time—that is, the time the Argon beam needs to remove the organic layer. The method does not give an exact value that can be related to a continuous measurement of thickness but is dependent on estimations of the quantity and quality of the ions that are sputtered off.

In our study and in the study of pellicle formation on permanent teeth (10) the same instrument and similar settings of the instrument were used. The two main differences observed are a slower initial build-up and a leveling out of the adsorption process at a thickness corresponding to one-third that of the pellicle on permanent enamel. The enamel pieces in the experiments of Skjørland et al. (10) were carried for 2 h in an appliance on the buccal surface of the teeth. If shearing forces influence the formation of the pellicle, they should have acted just as strongly on the enamel pieces worn in an appliance as they do on the enamel pieces placed in the buccal sulcus.

The TEM pictures of pellicle at various formation times corroborate the results of the Auger analyses (Fig. 3). The proteins are initially rapidly adsorbed to the enamel surface, but the formation seems to level off and results in a homogeneous dense layer after 2 h. Compared with the ultrastructural appearance known from TEM pictures of pellicle on permanent enamel, the second globular layer of the pellicle is not formed (9). This is in contrast to observations on permanent teeth in adults, where the pellicle has been described to consist mainly of globules (18). Lie (12) first described the ultrastructural appearance of the 2-h pellicle formed on hydroxyapatite splints in individuals with permanent dentition. The TEM pictures showed a predominantly globular pellicle, with globules varying between 25 and 125 nm in diameter. The initial pellicle was described as a relatively even coating with a homogeneous granular structure without any signs of globular components but with material resembling small buds (12). Lie's description of the initially formed pellicle (12) is in accordance with our observations of the ultrastructure of pellicle formed on deciduous enamel. In other studies concerning the aggregates or the globular part of the pellicle secretions with various protein contents have been used. With increasing protein content, the density of globular particles (micelle-like structures) increases (19). The protein content of whole saliva has been shown to increase with increasing age (20). It may therefore be possible that saliva from the children in this study does not contain enough protein to support the formation of aggregates (globules) making up the globular part of the 2-h pellicle.

The results of this study indicate that there are distinct differences between pellicle on primary teeth and pellicle on permanent teeth.

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