# Electron microscopic studies of human mixed saliva

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With foam components removed, mixed saliva from three donors were solidified in liquid nitrogen and sectioned, mounted, and fixed. Examination by transmission (TEM) and scanning (SEM) electron microscopy and energy-dispersive X-ray (EDAX) analysis were performed for paraformaldehyde-fixed sections, some of which were $OsO_4$-postfixed. The TEM and certain SEM examinations showed the presence of fine and dense salivary network structures, seemingly originating from the major fibrous components. In $OsO_4$-treated sections, TEM pictures showed reticulated arrangements with open cellular diameters down to 0.2 μm. The EDAX analyses particularly showed the presence of Ca, Fe, K, P, and S, with increased Ca readings in major components. Untreated sections showed that strands, with diameters of more than 1–2 μm, had more electron-dense central portions than peripheries and sometimes had interior, very electron-dense, granules. The observed features indicate that saliva has internal structures consistent with its colloid chemical characteristics. □ *Cryostat sectioning; EDAX analysis; scanning electron microscopy; transmission electron microscopy*

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A method has recently been presented for instantaneous solidification in liquid nitrogen and subsequent sectioning and staining for light microscopic examination of samples of mixed saliva and saliva fractions (1, 2). Using this method, Glantz et al. (2) demonstrated the presence of structural elements in fresh saliva and in saliva fractions from subjectively healthy humans.

At the light microscopic level all saliva samples showed an intrinsic microarchitecture dominated by (a) a loose overall filamentous network, frequently with approximately 120° angles between the structural components, and (b) collections of lipoid droplets. In the mixed saliva sections observations were also made of microorganism-like structures located in comparatively large bundles or bunches associated with epithelial cells and/or major, seemingly amorphous, salivary macromolecular strands. These same types of structure have also been identified in the sections from the donated salivary fractions.

Bearing in mind the possibility that saliva could have structural details not revealed at the light microscopy level, it was considered worthwhile to perform electron microscopic studies of similarly prepared thin sections of quick-frozen saliva.

## Materials and methods

Two to three milliliters of stimulated mixed saliva was donated during empty mouth chewing movements by three test persons: two men age 48 and 51 years and one woman age 20 years. All the test persons considered themselves to be in good general health and were dentate and in good oral health at general dental examinations. The samples were donated between 1000 h and 1100 h, and no food or drink had been consumed by the test persons during at least a 2-h period before sampling.

Within 5 min from the beginning of sampling multiple droplets of the non-foam-containing portion of the samples were placed, with minimum shear, into a thermoflask containing not less than 1000 ml of liquid nitrogen (−195.8°C). After about 5 min of immersion, the solid saliva samples were retrieved with a stainless steel tweezer from

the bottom of the thermoflask, immobilized with a special embedding medium for frozen specimens (type O.T.C. Compound, Ames Co., Elkhart, Ind., USA), and immediately transferred to cryostat microtome chucks. The chucks with their mounted frozen saliva samples were then transferred to a cryostat microtome (type AO Histostat Cryostat Microtome, AO Reichert Scientific Instruments, Buffalo, N.Y., USA). At a temperature of $-2 \pm 1$°C the samples were sectioned serially with a new knife and transferred to glass microscope slides precoated with a thin film of polyvinylbutyral resin (Butvar B-98 solution, Electron Microscopy Science, Fort Washington, Pa., USA).

The section-carrying microscope slides were stored in a deep freezer maintained at subzero temperatures, to enable sublimation of aqueous components to take place during 6–24 h. Thereafter they were moved to a silica-gel-containing storage desiccator, where final removal of moisture occurred at room temperature during an additional period of 20–30 h.

Finally, after paraformaldehyde vapor fixation for 90 min some Butvar coatings with their saliva sections were stripped from the slides via a water bath and quickly transferred to transmission electron microscope (TEM) grids (type TEM grids, 200 mesh). Other sections were postfixed for 2 h at room temperature with vapor from a 2% aqueous solution of $OsO_4$ before stripping and the final carbon coating for TEM examination.

The TEM examinations were performed in a Hitachi HS-8 instrument and the scanning electron microscopic (SEM) examinations in a Hitachi S-450 instrument, also equipped with an integrated energy-dispersive X-ray (EDAX) analyzer.

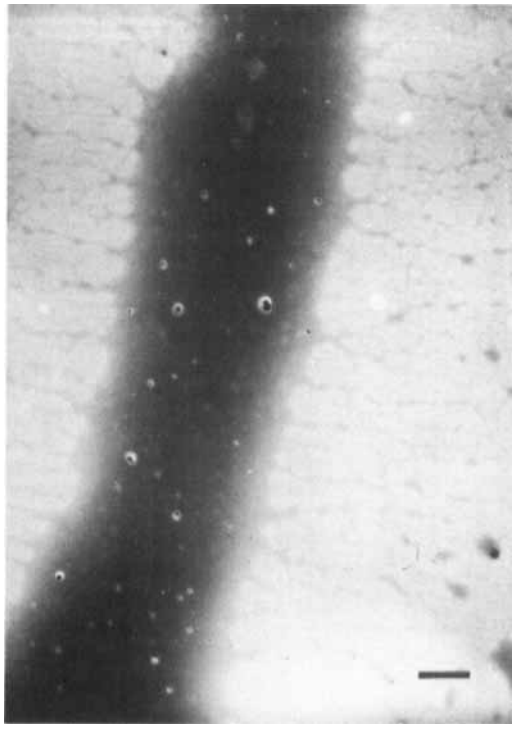

Fig. 1. Transmission electron micrograph of a paraformaldehyde vapor-fixed, approximately 10-μm-thick section of mixed saliva from a subjectively healthy 48-year-old male donor. The picture shows a major structural strand, with a somewhat varying electron density, from which a fine filamentous network is extending, often with round 120° angles between the network components. Some of the components seem to reunite into extended structures at a distance from the major strand. Length of bar = 1 μm.

## Results

The most important observations made at the TEM examinations are illustrated in Figs. 1–5 and are as follows:

1. Filamentous network structures were present in both the $OsO_4$-fixed and the non-fixed sections of all the examined samples. The structures differed somewhat but seemed to originate from the same major fibrous components (Figs. 1–4) first identified by light microscopy (1, 2). Finer fibers radiated into most parts of the sections, where they had both denser and looser connecting filamentous segments.

In many parts, especially of the $OsO_4$-fixed sections, the structures had a honeycomb- or foam-like arrangement (Figs. 4 and 5). The specimens showing these lamellar structures were taken from the center portions of the samples at relatively great distances from any surface foam that might have accidentally accompanied the samples when first immersed in liquid nitrogen. The sizes of the individual foam-like structures were noted to

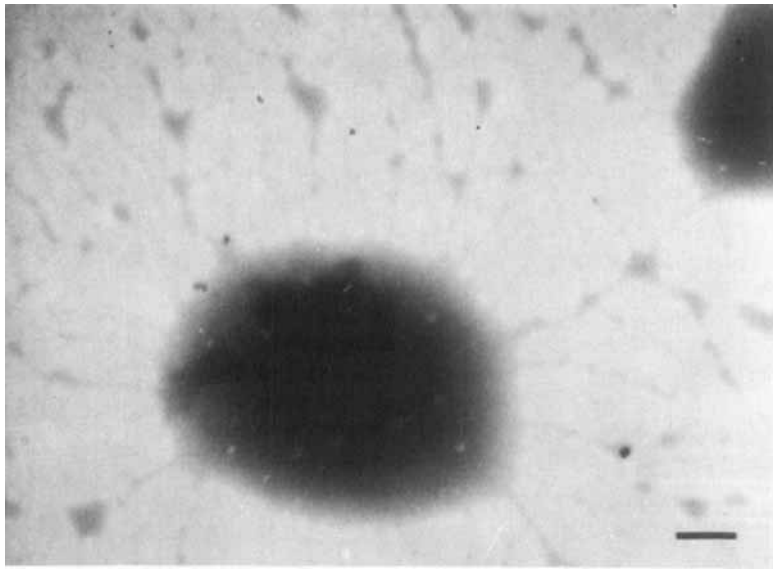

Fig. 2. Transmission electron micrograph of a paraformaldehyde vapor-fixed, approximately 10-μm-thick section of mixed saliva from a subjectively healthy 48-year-old male donor. The picture shows a round major structural component with a somewhat varying electron density. From this component a fine network structure is extending, some of whose fine components reunite into larger pools of material at a distance from the major component. Length of bar = 1 μm.

vary considerably, with observed minimum diameters as small as 0.2 μm (Fig. 5).

2. In the non-$OsO_4$-fixed sections fibrous structural components with diameters greater than 1–2 μm were observed to have somewhat more electron-dense center portions than peripheries (Figs. 1–3).

3. In some non-$OsO_4$-fixed sections major structural components sometimes included rounded structures with a much higher electron density than that observed for the surrounding, continuous structures themselves (Fig. 3).

The results of the SEM studies were less

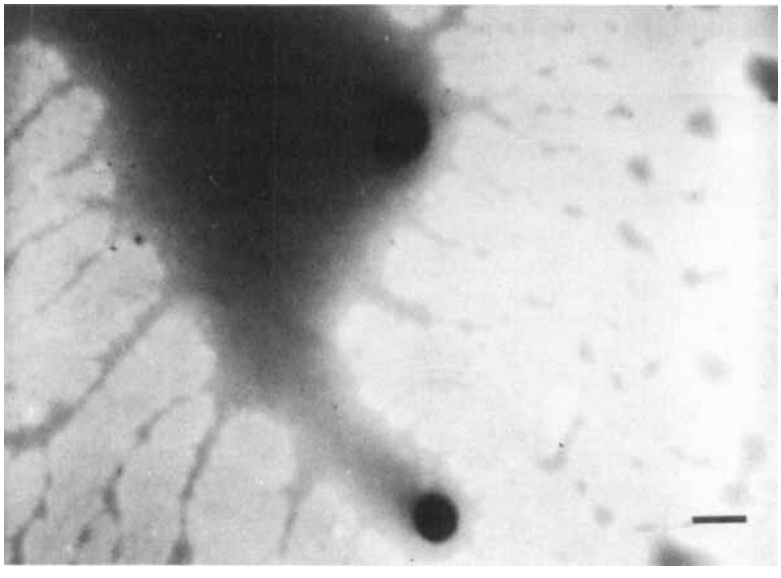

Fig. 3. Transmission electron micrograph of a paraformaldehyde vapor-fixed, approximately 10-μm-thick section of mixed saliva from a subjectively healthy 48-year-old male donor. The picture shows a rounded, somewhat irregularly shaped, major structural component with a varying electron density and extending branches of material, which further divide into a finer network structure. At a distance from the major component, some of the fine structures reunite into larger pools of material. Note especially the two rounded, very electron-dense objects that can be seen inside the major structure in the vicinity of its periphery. Another much smaller such object can also be observed close to the end of the side branches. Length of bar = 1 μm.

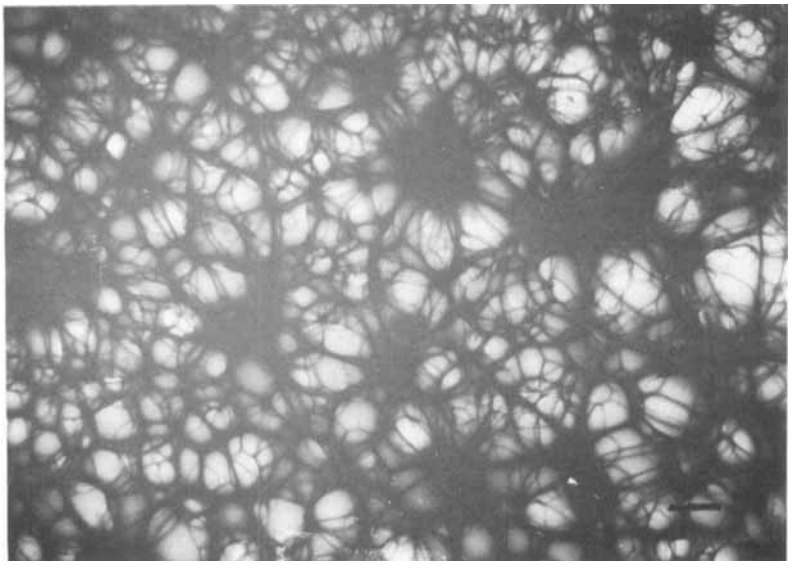

Fig. 4. Transmission electron micrograph of a paraformaldehyde vapor-fixed and $OsO_4$ vapor-postfixed, approximately 10-μm-thick section of mixed saliva from a subjectively healthy 51-year-old male donor. The picture shows a somewhat irregular honeycomb or foam-like network structure, created by structural components of various dimensions. Length of bar = 1 μm.

detailed than those from TEM. Some interesting observations were made, however, including the one that wart-like or cut-branch-like extensions could be observed on the surfaces of some exposed major structural components (Figs. 6 and 7).

The most important results of the EDAX analyses are given in Figs. 8–11. For reference, Fig. 11 gives the EDAX spectrum of the surface of the used type of carbon-coated specimen holder. As can be seen in Figs. 8–10, various amounts of Fe, P, S, Ca, and K were recorded with increased Ca signals in the areas of the major structural components of the saliva sections.

## Discussion

Saliva plays many key functional roles in

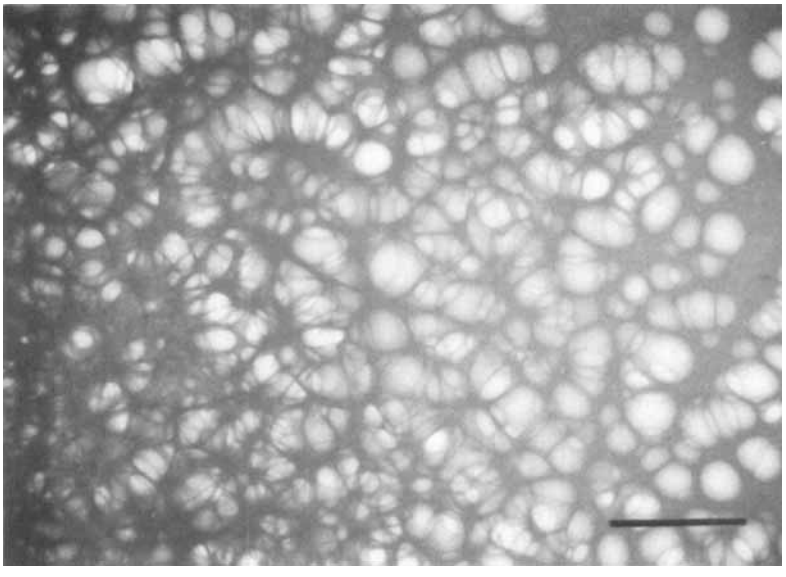

Fig. 5. Transmission electron micrograph of a paraformaldehyde vapor-fixed and $OsO_4$ vapor-postfixed, approximately 10-μm-thick section of mixed saliva from a subjectively healthy 51-year-old male donor. The picture shows a relatively dense network structure with honeycomb or foam-like appearance. Length of bar = 1 μm.

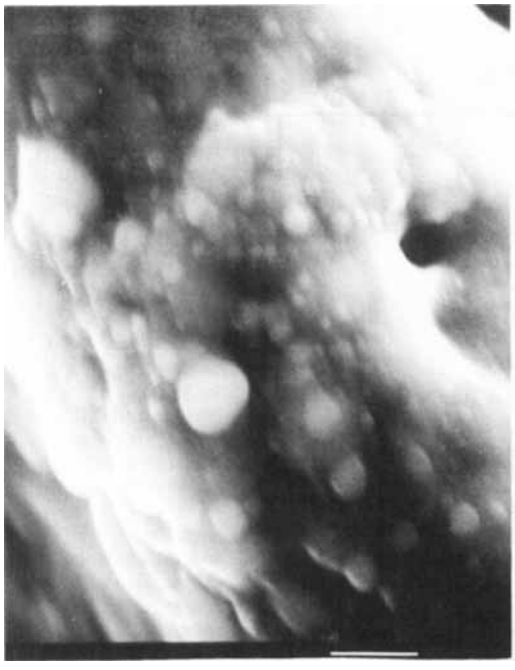

Fig. 6. Scanning electron micrograph of the surface of a paraformaldehyde vapor-fixed and $OsO_4$ vapor-postfixed, approximately 10-μm-thick section of mixed saliva from a subjectively healthy 20-year-old female donor. The picture shows a major structural component with multiple wart-like surface extensions. Length of bar = 15.6 μm.

Fig. 7. Scanning electron micrograph of the surface of a paraformaldehyde vapor-fixed and $OsO_4$ vapor-postfixed, approximately 10-μm-thick section of mixed saliva from a subjectively healthy 20-year-old female donor. The picture shows rounded, wart-like, surface extensions with interconnecting strands of smaller dimensions. Length of bar = 7.8 μm.

the maintenance of so-called normal oral functions. As has been pointed out, many of these functions would be efficiently served if saliva had an internal structure. The recent development of a method for light microscopic studies of saliva (1, 2) showed the presence of several such salivary structural features, including an overall, seemingly weak, filamentous network structure and local oil-in-water like arrangements of dispersed lipoid droplets.

The results of the light microscopic examinations of saliva samples, however, also made it clear that the structures of mixed saliva were highly complex and might include structural components or interactions even at the ultrastructural level. It was therefore thought worthwhile to study saliva structures also by transmission and scanning electron microscopic examinations.

Even though the methods have not previously been used for electron microscopic studies of saliva, they are based on a series of well-documented methodologic steps and stages. It is therefore not likely that they could, either separately or in combination, have created the observed fine salivary structures.

The examined saliva sections had a thickness of about 10 μm, to enable early evaluation of the main compositions of the sections and the possible presence of three-dimensional structures. Successful such analyses were made (Figs. 8–11), and a three-dimensional structure could also be observed (Figs. 1–5). At the same time, however, by studying these comparatively thick sections, the resolution of finer structures was limited. This was especially true inside the major structural components, and thin-

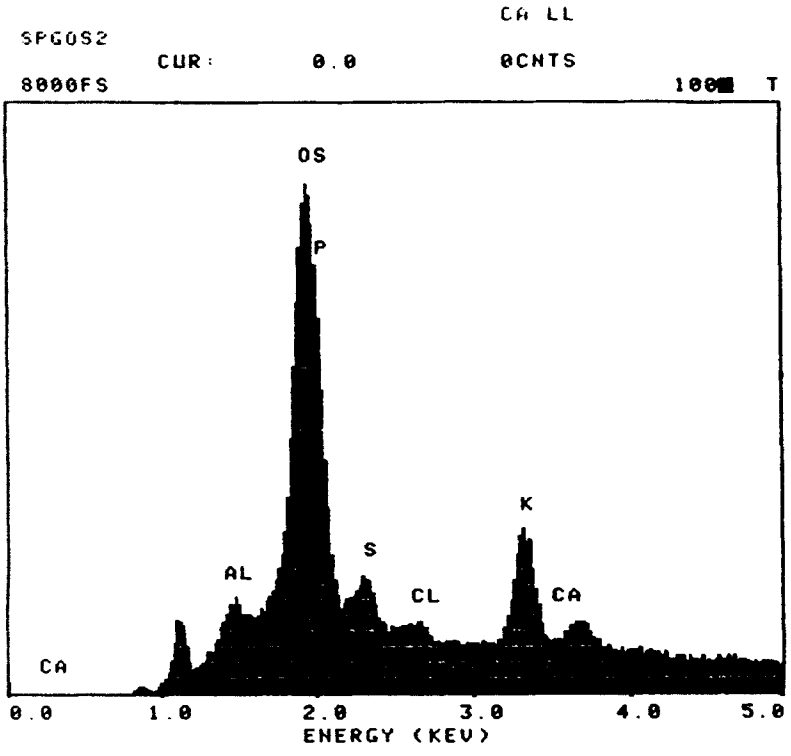


Fig. 8. Energy-dispersive X-ray (EDAX) spectrum of a paraformaldehyde vapor-fixed and $OsO_4$ vapor-postfixed, approximately 10-µm-thick section of mixed saliva from a subjectively healthy 20-year-old female donor. In SEM, this section had only a few structural characteristics. When signals from background (Al) and staining (Os) materials are excluded, this spectrum indicates the presence of relatively large amounts of P and, to a certain extent, also of K in the section. Detectable amounts of Ca, Cl, and S were also noted.

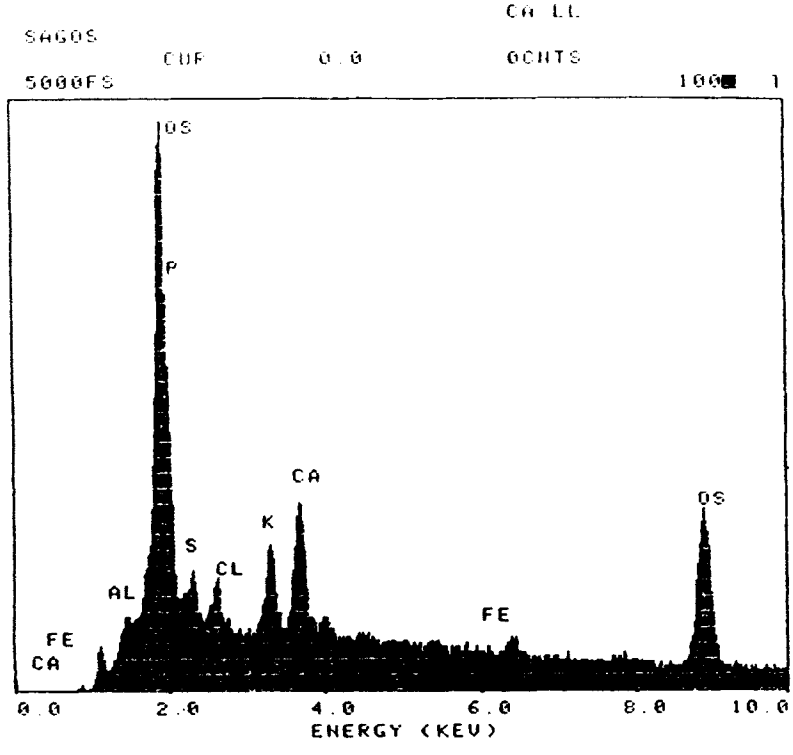


Fig. 9. Energy-dispersive X-ray (EDAX) spectrum of surface shown in Fig. 6. In comparison with the spectrum given in Fig. 8, this shows increased amounts of Ca and also the presence of Fe.

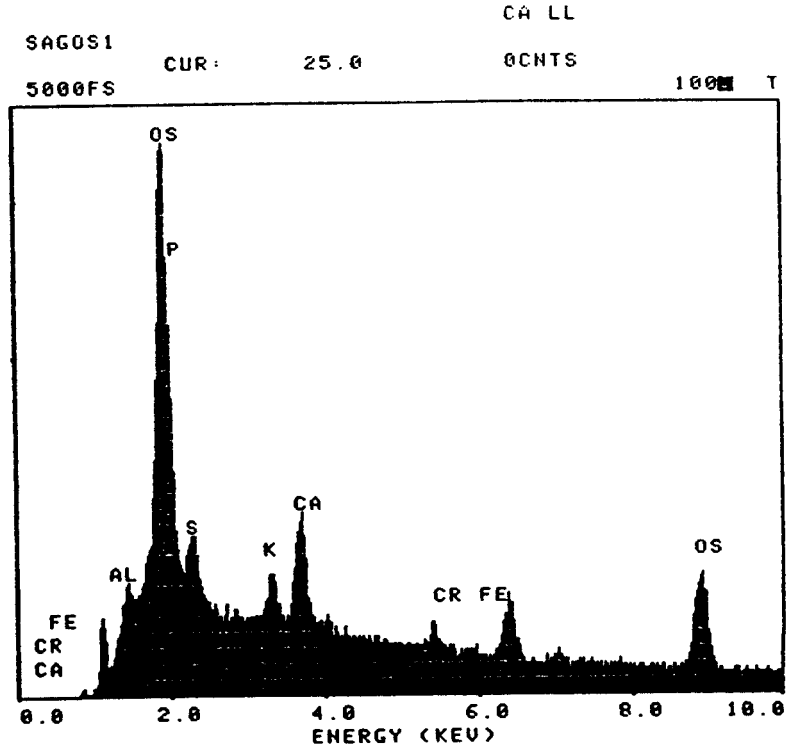


Fig. 10. Energy-dispersive X-ray (EDAX) spectrum of surface shown in Fig. 7. In general, this spectrum resembles the one given in Fig. 9. Even higher Ca and Fe peaks were observed, however, and also the presence of Cr, but not of Cl.

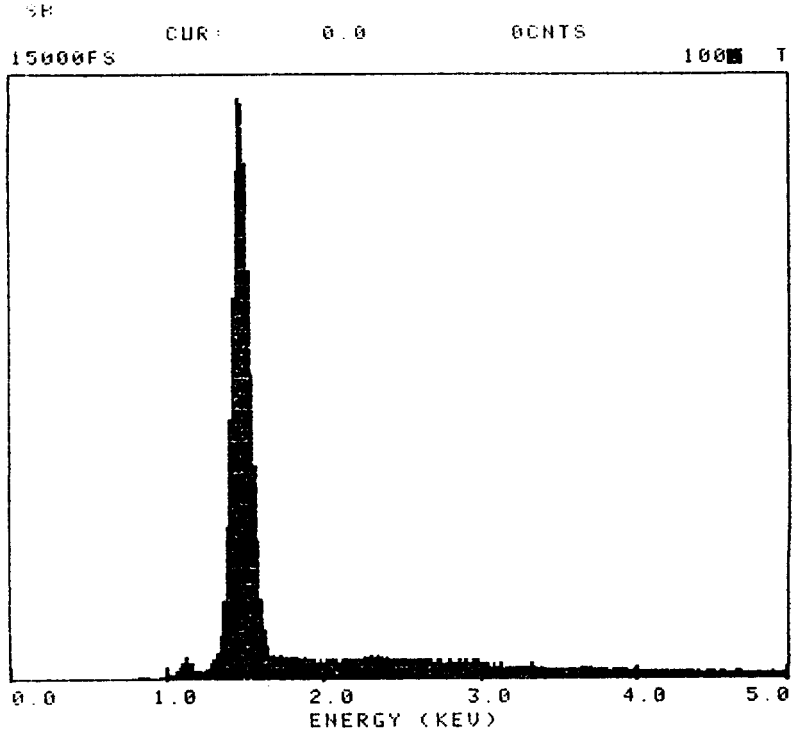


Fig. 11. Energy-dispersive X-ray (EDAX) spectrum of surface of carbon-coated type of specimen holder used in obtaining EDAX spectrums presented in Figs. 8–10.

ner sections must now be prepared to improve resolution at the ultrastructural level.

The overall main observations made at the thick-section TEM level confirm those made during light microscopic examinations (2). In addition, we also observed a fine elaborate network structure stretching from the central major salivary fibers, through intermediary filamentous connectors, to even finer peripheral structures, which, especially in the $OsO_4$-fixed sections, sometimes had an almost honeycomb- or foam-like organization (Figs. 4 and 5).

A central observation was that in non-$OsO_4$-fixed sections there were several rounded and very electron dense objects within some of the major scaffolding elements (Fig. 3). They may have a different origin from the fibrous network itself, and it is possible that they were entrapped within the network components as a consequence of some physiologic function of the network.

The EDAX analyses showed the presence of relatively large amounts of calcium in the areas of the major components of the salivary sections. This finding could perhaps be explained by the documented ability for calcium association frequently observed for certain proline-rich salivary proteins (for a review, see Bennick (3)).

No definite observations were made of the lipoid droplets seen at the light microscopic level. This could be due to several facts, including the relatively low volumetric concentration of these droplets and the lack of lipid-specific stainings/fixations without influence on surrounding salivary material.

On the basis of mathematical calculations from data obtained in experimental studies of the time-dependent rheologic behavior of mixed saliva, Glantz & Friberg (4) predicted the existence of a dominating structural component in saliva. The filamentous network structure visualized in detail for the first time in this study is of the type predicted earlier (4). It is probably also responsible for many of the rheologic characteristics of saliva.

The precise functions of the noted fibrous network can, of course, not be stated at present. It is, however, likely that it is of significance in the lubrication of both hard and soft oral surfaces and for the clearance from the oral cavity of food particles, desquamated epithelial cells, microorganisms, and similar objects. The dimensions of some of the network filaments observed are, in fact, so small (about 0.2 μm) that not even single microorganisms could avoid association with them.

The mechanical stability of the observed network structure is obviously small, as demonstrated by the low viscosity of saliva and its pronounced non-Newtonian behavior (3, 5–8). It is, however, also at least partially thixotropic, which means that after mechanical breakdown it has a certain ability to reconstitute itself during a period of stress removal.

Structures with the observed nature usually have colloidal characteristics and should therefore behave at least partially in accordance with known colloid chemical lines of reaction. This means, for example, that the structure-forming salivary component/components must reach a certain critical minimum concentration before the actual network begins to arrange itself. In this context, it is therefore interesting to note that the so-called critical micellar concentration of human mixed saliva has recently been found to be around 10% in aqueous solutions (J. Sefton. Personal communication, 1987). It is also at this approximate saliva concentration that a general colloid chemical behavior has been observed for saliva and suspended microorganisms during studies of the relative colloidal stability of suspensions of streptococci in saliva from 'heavy' and 'light' plaque formers (9). The noted differences between these groups of patients might therefore, at least partially, be related to differences between the colloidal properties of the individual saliva network structures.

The studies performed do not yet tell which particular salivary components are involved in the formation of salivary microstructural networks. Bearing in mind the known composition of saliva (10), it is, however, likely that the salivary glycoproteins with their documented rapid film-forming ability (11) play a significant role in the process. Dominance of such a compositional component is consistent with the observed

effect of the postfixation of the sections with $OsO_4$ vapor. Especially in the major structural fibers there can, however, also be other components, as indicated by the above-described variation in the electron density of included granules. It will be necessary to evaluate saliva in even thinner sections to provide the answer to that question. Such studies are in progress.

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