Structural studies of human saliva

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Samples of mixed saliva and of parotid and sublingual/submandibular saliva fractions from four donors were subjected to instantaneous solidification in liquid nitrogen followed by sectioning in a microtome/cryostat. The sections were stained with hematoxylin-eosin, periodic acid-Schiff, Alcian blue, Oil-red-O, or Sudan Black B and then examined at the light-microscopic level. In all the sectioned samples several previously never described features were observed, the most pronounced of which were a loose overall network structure and collections of lipoid droplets often in a loose arrangement. In the mixed saliva sample sections many of the microorganism-like structures were observed in large bunches associated with epithelial cells and densely staining saliva components. The present method was tested in a series of experiments for possible errors. □ *Cryostat sectioning; light microscopy; saliva*

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It is generally accepted that saliva plays several significant roles in the maintenance of essential oral functions, including lubrication, water balance, chewing, taste sensations, and clear speech. These functions are, in their turn, partially or totally based on a range of physicochemical, biochemical, antimicrobial, and/or biophysical activities in saliva which have been reviewed previously (1). These and other physiologic properties of saliva, particularly those associated with the continuous lubrication of oral biosurfaces, restorations, and foodstuffs, would be enhanced by a fluid possessing an ordered structure such as found in a weak gel.

Observations of the presence of elastic threadlike components in saliva were made many years ago and include those by Fano (2), Aggazotti (3), Colombini (4), and Kaiser-Nielsen (5). Furthermore, the presence of a structural viscosity in human saliva was described by Hoepfner (6) as early as 1943; Hoepfner's findings have since been verified in several more recent studies (7–11). A truly thixotropic behavior in certain mixed saliva samples has been reported by Glantz & Friberg (7), who suggested that this property could, under certain circumstances, be related to a single structural component.

The non-Newtonian rheologic behavior of human saliva is well established. However, few reports have been found in the literature describing the actual micromorphology of fresh saliva or saliva fractions. The comparatively rapid enzymatic structural breakdown that occurs in saliva prevents the use of most of the common methods for structural studies of fluids. Of the few morphologic studies found in the literature, most have been performed on smears of acid-treated saliva, in which precipitation of mucin and mucin-containing saliva components has already taken place (5).

More recent investigations of the rheologic properties of saliva (7, 11) have indicated the possibility that the frequently observed threadlike components are not the only structural elements in saliva. Since the development of more effective saliva substitutes would be facilitated if their total structural requirements were better known, it was considered worthwhile to develop new methods for more complex structural studies of fresh human mixed saliva and saliva fractions.

Materials and methods

Two to three milliliters of stimulated mixed saliva was donated during empty-mouth chewing movements by four test persons: two men (A, B) aged 32 and 51 years and two women (C, D) aged 20 and 32 years. All the test persons considered themselves to be in good general health and were fully dentate and in good oral health at general dental examinations. The samples were donated between 0900 h and 1100 h, and no food or drink had been consumed by the test persons within at least 60 min before sampling. From test persons A and D approximately 2 ml of parotid and submandibular/sublingual saliva fractions were also obtained, using the methods described by Curby (12) and Block & Brotman (13).

Within 5 min of the sampling of the mixed saliva and not more than 20 min after the sampling of the saliva fractions multiple 0.01- to 0.1-ml droplets of the non-foamy portions of the samples were poured with minimum shear into the opening of a thermoflask containing not less than 1000 ml boiling liquid nitrogen (−195.8°C). Within 2 min of immersion the then solid saliva samples were retrieved from the bottom of the thermoflask with stainless steel tweezers. The solid pieces of saliva were then placed on a cryostat/microtome stage (type AO, Histostat Cryostat Microtome, AO Reichert Scientific Instruments, Buffalo, N.Y.) and immobilized with special embedding medium for frozen tissue specimens (type O.T.C. Compound, Ames Co., Elkhart, Ind.). The stage and sample were immediately transferred to a cryostat microtome (type AO), where it was kept at −21 ± 1°C until sectioned. Each specimen was sectioned in series with a new knife and then mounted on acid/alcohol- and acetone-washed glass slides. Finally, the serial sections were stained at room temperature using the following staining methods (14): 1) hematoxylin-eosin; 2) periodic acid-Schiff (PAS) (with and without digestion); 3) Alcian blue (pH 1.0 and 2.5); 4) Oil-red-O; and 5) Sudan Black B.

The specimens were covered with glass microscope cover slips, sealed with varnish, and examined in a light microscope (type AO, Series 10, Microstar).

Studies of some possible errors of the method

Freezing of tissues is frequently used without inducing major negative structural effects both in pathology and in food technology. From a theoretic point of view, there are always concerns about modifications of internal structures in frozen fluids, especially as the result of impurities or contaminations. To a certain extent, these risks were evaluated in the following experiments:

1. Triply distilled water was handled in exactly the same manner as the saliva samples. The subsequent microscopic examinations of the frozen and stained sections did not reveal the presence of any type of detectable structure or objects on the microscope slides. On the basis of this result, it was concluded that the *handling* of the saliva samples was not likely, by itself, to have induced any of the observed saliva structures.

2. The possible influence of different relative amounts of water in the saliva samples on the observed overall structures was studied by mixing equal volumes of triply distilled water and whole saliva from one of the test persons before freezing, sectioning, and staining.

The microscopic examination of the diluted specimen frozen sections did show structural components of the same kind as those observed in the concentrated samples. On the basis of this result, it was concluded that it was not likely that the observed saliva structures were induced as the result of any concentration effects created by water-soluble or water-miscible saliva components.

3. The possible influence of segregation of non-polar, non-hydrogen-bonding constituents on the observed saliva structures was studied by vigorous mechanical mixing, for about 60 sec, of equal volumes of double-distilled hexane and whole saliva (from one of the test persons) immediately before freezing, sectioning, and staining.

The microscopic examinations of these

sections showed that almost complete phase separation had taken place at the time of the liquid–solid transition. In parts of the sections, however, translucent droplets (probably formed by hexane or hexane-containing material) could be identified in the saliva phase of the Oil-red-O-stained sections. The dispersed droplets did not seem to have produced any noticeable interactions with the continuous, probably hydrophilic, saliva phase but were occasionally found to be associated with the lipoid droplets. On the basis of the results of this experiment, it was concluded to be unlikely that observed saliva structures were created by some induced concentration effect from water-insoluble or water-immiscible contaminants or impurities.

Results

On the basis of the light microscopic examinations of the stained whole saliva sections the following major observations were made:

In most areas of the hematoxylin–eosin-stained sections a homogeneous background structure was shown to create a network often with angles of approximately 120° between the network components (Figs. 1 and 2). In addition, these sections showed bands or tracks of exfoliated epithelial cells also containing microorganism-like structures and exhibited interconnecting strands of seemingly amorphous material with fewer inclusions and microorganism-like structures. Most of the microorganism-like structures were cocci-shaped and located in comparatively large bundles or bunches, which were morphologically associated with the epithelial cells and/or the more heavily stained portions of the observed seemingly amorphous structure components (Fig. 2).

The PAS-stained sections showed the same structural elements, including the strands and the seemingly amorphous components. After digestion these amorphous structure components and strands were not detected.

Both of the Alcian blue-stained sections (pH 1.0 and 2.5) showed the same types of strands and seemingly amorphous material as the hematoxylin-eosin and the PAS sections (before digestion) (Fig. 3).

The Oil-red-O and Sudan Black B stainings both showed collections of varied-sized lipoid droplets in many parts of the sections.

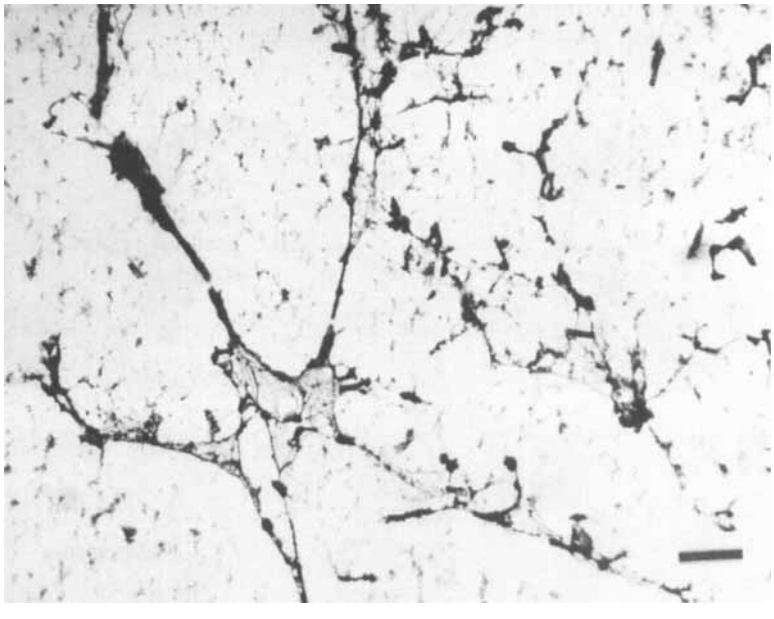

Fig. 1. Hematoxylin-eosin-stained section of whole saliva from a 32-year-old subjectively healthy female donor. The section shows the presence of a network created by seemingly amorphous structural components of various dimensions. Many of the network components meet at approximately 120° angles, and the figure also shows the presence of epithelial cells in contact with the network. Length of bar = 1 mm.

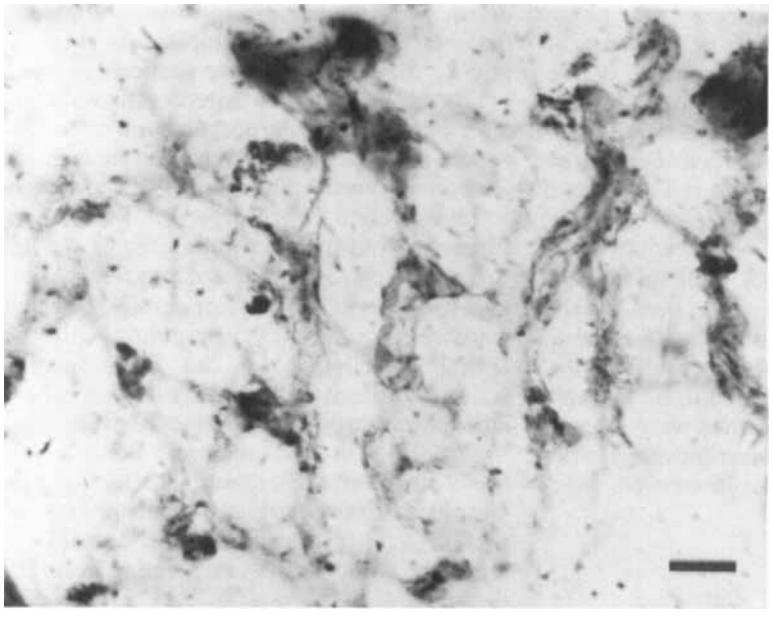

Fig. 2. Hematoxylin-eosin-stained section of whole saliva from a 20-year-old subjectively healthy female donor. The section shows the presence of fine network structures created by seemingly amorphous components of various dimensions. The network structures are associated with microorganism-like particles and epithelial cells. Length of bar = 10 μm.

Many of the larger droplets were noted within or close to the cell clusters or the seemingly amorphous structure components. Similar types of lipoid droplets were also frequently observed in the more homogeneous parts of the sections with less background staining (Fig. 4). On storage, especially the larger lipoid droplets were found to move closer to the cover slip and slightly out of focus from the rest of the sections.

On the basis of the light microscopic examinations of the stained and sectioned samples of parotid saliva, the following observations were made:

An overall loosely arranged structure was

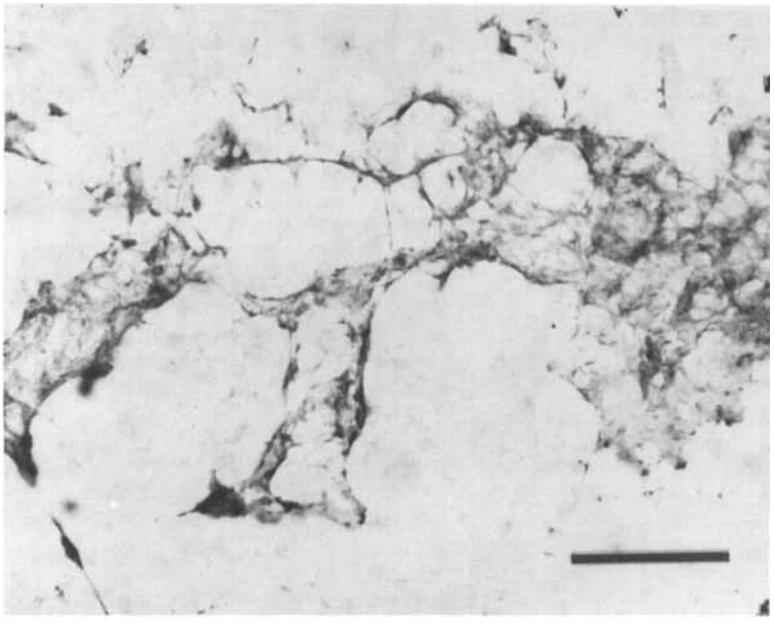

Fig. 3. Alcian blue-stained section (pH 1.0) of whole saliva from a 51-year-old subjectively healthy male donor. The section shows the presence of a somewhat coarse network structure created by strands of seemingly amorphous material. Length of bar = 1 mm.

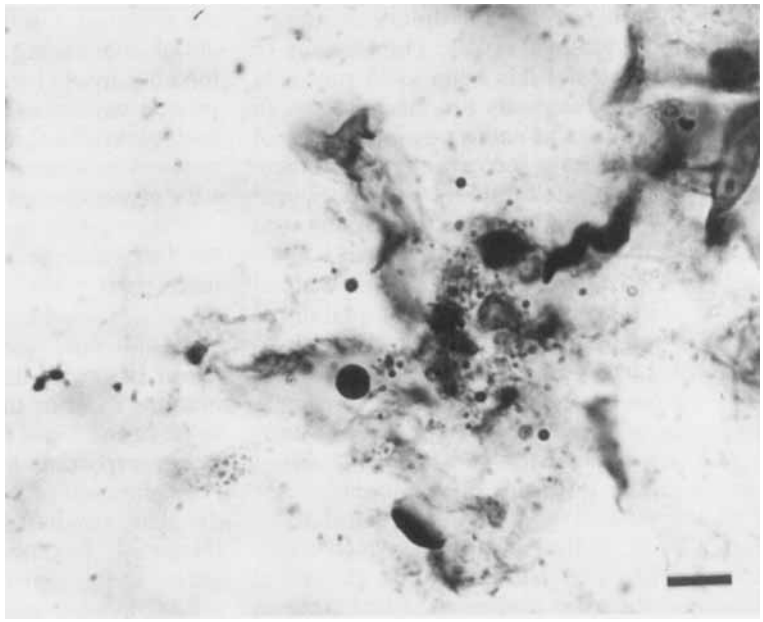

Fig. 4. Oil-red-O-stained and hematoxylin-counterstained section of whole saliva from a 20-year-old subjectively healthy female donor. The section shows the presence of multiple lipoid droplets of various dimensions, bunches or bundles of microorganism-like particles, and epithelial cells. Length of bar = 10 μm.

observed in the hematoxylin-eosin-stained sections. It was similar to some of the structures observed in the whole saliva sections but lacked epithelial cells and microorganism-like components.

Loosely arranged groups or bands of lipoid droplets were observed in the Oil-red-O- and Sudan Black B-stained sections, although not with the same frequency as in the corresponding whole saliva sections.

On the basis of the light microscopic examinations of the stained and sectioned samples of submandibular/sublingual saliva the following general observations were made:

Against a poorly stainable homogeneous background groups of small lipoid droplets were observed, often in a loose chain-like arrangement similar to that noted for the whole saliva sections.

Discussion

Saliva plays an essential role in many key oral functions. The significance and multiplicity of these functions is perhaps most easily understood by the complexity and severity of the clinical symptoms associated with xerostomia or 'the dry mouth syndrome' (15).

Details known about the functions of saliva are particularly those related to biochemistry and microbiology. Although also clinically important, comparatively less knowledge seems to be available about other, mainly biophysical, saliva functions such as friction and wear protection and diffusion and spreading of saliva, particularly into narrow gaps and spaces.

The lack of detailed understanding of the morphologic structure of this non-Newtonian fluid was the incentive for the development of the presented method.

As such, the method is based on an instantaneous liquid–solid phase transition of saliva at a temperature (−195.8°C) that is much below the freezing point of this fluid. The growth of large ice crystals is thus prevented, and, consequently, the destruction of possible mechanically weak intrinsic salivary components is minimized.

Immersion of saliva or other body fluids into liquid nitrogen for structural investigations has, apart from a recent abstract from our group (16), not been found to have been reported previously. It is, however, not

likely that this procedure should cause any major methodologic errors. The freezing of hydrated material has been used routinely in pathologic diagnosis for many years. In addition, studies of some possible errors of the method clearly indicate that immersion of saliva drops into liquid nitrogen does provide a valid method for preserving the microarchitectural structures in saliva.

Freezing of pieces of tissue in small metal vessles with propane in tanks of liquid nitrogen is often favored over direct liquid nitrogen freezing. The reason is that propane and similar cryogens do not have the same tendency as nitrogen to form thermo-insulating gas films on the surface of immersed specimens. In this study open liquid nitrogen freezing was still used, mainly because of the initial state of the specimens, which made them difficult to handle with a technique designed for solid specimens. Furthermore, no negative effects from direct liquid nitrogen immersion were observed. If, indeed, gas bubbles were created, they seemed rapidly to disperse from the saliva specimens and move to the surface of the liquid nitrogen as the specimens were sinking to the bottom of the flask. Attempts will, however, still be made to develop more accurate freezing methods also for saliva and other biofluids.

The methods used for mounting, sectioning, and staining of the frozen saliva samples are all well established in histologic and pathologic research and clinical diagnosis. Although they may be associated with some methodologic problems, none of them has been reported to introduce the kind of structures observed in the frozen saliva sections.

When Figs. 1–4 are examined, it is evident that human saliva is a rather structured biologic material, probably better classified as a fluid tissue than a solution. These figures show that there is an overall fine, filamentous network structure throughout the samples. This network often seems to have angles of approximately 120° between the individual structure components. This is an interesting observation, as it is in accordance with the structures of many colloidal networks (17). At this stage of investigation it is not possible to state the exact chemical nature of the network or the strands of seemingly amorphous material. On the basis of the knowledge about the average composition of human mixed saliva (1) and that of morphologically related structures in other biologic and nonbiologic systems, it is likely that the observed network is formed, at least partially, by salivary glycoproteins.

Staining with specific techniques for lipids showed multiple, small lipoid droplets in many parts of the samples. The sizes of these droplets varied somewhat, but their diameter did not, however, seem to exceed about 10 μm. In the light microscopic examinations most of the lipoid droplets seemed to be in the 1-μm range, often with a chain-like interparticle arrangement. As the hexane treatment of the saliva, at least for some droplets, resulted in the formation of round bilayered hexane–lipid structures, there seems to be a strong tendency for hexane–lipoid droplet association but not for the droplet material to dissolve in hexane. This finding suggests that at least some of the original saliva lipoid droplets contained the type of long-chain unsaturated fatty acids reported to be present in saliva (18).

These structures and their respective dimensions suggest that one of the structural features of human saliva is that it is an oil-in-water type of emulsion or microemulsion. This conclusion is further supported by the observation of a tendency, on storage, for phase separation especially between larger lipoid droplets and their immediate surrounding tissues.

At present it is not possible to state anything definite about the key physiologic function of the observed emulsion structure and its components. It should be kept in mind, however, that emulsions can be well suited to fulfill a range of both biophysical and biochemical functions (19)

In the mixed saliva samples another noted structural feature was the extraneous biologic material, such as epithelial cells and microorganism-like particles, observed in abundance. Most of the identified epithelial cells probably were naturally desquamated, as many contained intracellular lipoid material. It also was noted that neither the observed epithelial cells nor the microorganism-like particles were evenly distributed

in the samples. Rather, both the epithelial cells and most of the microorganism-like particles co-aggregated and were associated with the streaks of seemingly amorphous filamentous salivary material, at least when observed at the light microscopic level.

According to the PAS-stained sections and the Alcian blue-stained sections at pH 1.0 and 2.5, these streaks contained highly polar materials, possibly including those having sulfate and, perhaps, phosphate groups. The significance of charged salivary molecules for microbiologic aggregation in saliva has been stressed in many studies (20–25).

The observation of a microarchitecture in saliva is probably of considerable clinical significance. This property may produce the basis for a better understanding of such essential biologic phenomena as the clearance of microorganisms from the oral cavity and microbial adhesion to epithelial cells and to various types of oral solid surfaces. It is likely that the described method for structural studies of both whole saliva and saliva fractions can provide a tool for a better understanding also of other properties and functions of human saliva. In principle, there are few limitations to what pretreatment the saliva samples can be submitted to before liquid nitrogen immersion. The frozen sections of saliva also seem to enable application of almost any analytic histologic or histochemical technique. Many such studies are now in progress.

Finally, the described method should also be useful for structural studies of other biologic fluids and secretions.

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