Studies on the Glycolipids of Human Saliva and Gastric Juice*

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It has been reported that both human saliva and gastric juice (cf. Slomiany, B. L., and Slomiany, A. (1980) in Cell Surface Glycolipids (Sweeley, C. C., ed) American Chemical Society Symposium, No. 128, pp. 149-176, American Chemical Society, Washington, D.C.) contain substantial amounts of certain members of a series of novel glucoglycerolipids with a 1-O-alkyl glyceryl ether backbone. We have analyzed the glycolipids present in samples of saliva obtained from 10 individuals and in samples of gastric juice obtained from 5 individuals. In both fluids, compounds corresponding in the properties studied to standards of glucosyl- and lactosylceramides were found to be the major glycolipids. Other more complex glycosphingolipids were also present in smaller amounts. Human saliva was found to contain two glucoglycerolipids that were not detected in gastric juice. Analyses of these compounds indicated that they were mono- and diglucosyl diglycerides and were probably of bacterial origin. Methanalysis of the glycolipid fractions of saliva and gastric juice failed to reveal the presence of any more than traces of 1-O-alkyl glyceryl ethers. Our results do not exclude the possibility that glycerol ether-containing glucoglycerolipids occur in human saliva and gastric juice. However, at most they would appear to be rather minor components of either fluid.

A number of novel glyceryl ether-containing glucoglycerolipids have been reported to be present in human saliva (1), in human gastric juice (1-5), in human tracheobronchial secretions (6), and also in the gastric mucosal barrier of the rat (7). The majority of these reports has been reviewed (8). Because of the continuing interest of our laboratory in glyceryl ether-containing glycosphingolipids (9), we have attempted to isolate the major members of the above class of compounds from human saliva and human gastric juice. We describe here the results of these studies. Our findings differ substantially from those reported previously by others (8). The principal glycolipids present in both human saliva and gastric juice were found to be simple glycosphingolipids. Two glucoglycerolipids were detected in human saliva; these were mono- and diglucosyl diglycerides, which do not contain glycerol ethers. Evidence is adduced indicating that these particular compounds were derived from bacteria. Glucoglycerolipids were not detected in the samples of human gastric juice that were analyzed.

* Portions of this paper (including "Experimental Procedures," part of "Results," and Fig. 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-1215, cite authors, and include a check for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

† Glucosyl- and galactosylceramides were both employed as chromatographic standards during the course of these studies. They migrated as double bands and exhibited overlapping chromatographic migrations. In the chromatograms shown here, galactosylceramide was employed as the standard for a monoglucosylceramide. For this reason, the chromatographic migrations of the species of monoglucosylceramide detected are described with reference to the migration of this particular glycolipid. However, as shown under "Results," the monoglucosylceramide species present in the samples analyzed often migrated as three bands (e.g. Fig. 1), the first band migrating just ahead of the faster migrating species of the standard galactosylceramide and the third band migrating just behind the slower migrating species of galactosylceramide. As discussed in the text, the results obtained in this study indicated that glucosylceramide was the principal species of monoglycosylceramide, with lesser amounts of galactosylceramide also generally being present.

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Fig. 1. Results of analyses by TLC of various samples of human saliva. A: 1-3, the glycolipids of samples of whole saliva from three different individuals; 4, a mixture of standards of galactosylceramide, lactosylceramide (trace), GL-3, and GL-4; 5, standards of galactosyl- and sulfatoxygalactosylceramides. B: 1, the glycolipids of one sample of whole saliva; 2, the glycolipids of the supernatant of the same sample of whole saliva; 3, the glycolipids of the pellet of the same sample of whole saliva. C: 1, the glycolipids of one sample of whole saliva; 2, the glycolipids of the same sample of whole saliva, but treated with mild alkali; 3, a mixture of standards of galactosylceramide, lactosylceramide, GL-3, and GL-4; 4, standards of galactosyl- and sulfatoxygalactosylceramide; 5, standards of galactosylalkylacylglycerol and sulfatoxygalactosylalkylacylglycerol, treated with mild alkali; 6, standards of the two previous lipids, untreated. The glycolipid fractions of whole saliva and of the other samples shown were obtained by elution with acetone:methanol (9:1, v:v) (omitting prior elution with acetone) from glass columns loaded with silicic acid. The glycolipids shown in channels 1-3 of A were obtained from approximately 50-ml portions of samples of whole saliva. For fractionation of saliva by centrifugation (B), 60 ml of whole saliva was centrifuged at 12,000 × g for 30 min. The supernatant and pellet were lyophilized, subjected to lipid extraction, and then fractionated by column chromatography using silicic acid. The entire volumes of the acetone:methanol (9:1, v:v) fractions obtained from the pellet and the supernatant were then applied to the origin of the thin layer chromatogram. The solvent system used for TLC was chloroform:methanol:water (65:25:4, by volume). The chromatograms were stained with the aniline/diphenylamine reagent. The origins of the chromatograms are indicated by OR and the direction of migration is indicated by the arrow in the right-hand margin of C. The black dot in the right-hand margin of channel 1 of C indicates the slow migrating, alkali-labile glycolipid that has not been characterized. The numbers I-VIII in the right-hand margin of channel 2 of A indicate the eight glycolipid zones that were purified by preparative TLC and partial characterization of which was performed by GLC and other analyses (see text). The numbers I, V, and VI in the right-hand margin of channel 1 of C indicate the three of these eight glycolipids that were sensitive to treatment with mild alkali. The abbreviations used are: GC, galactosylceramide; SGC, sulfatoxygalactosylceramide; GG, galactosylalkylacylglycerol; SGG, sulfatoxygalactosylalkylacylglycerol.
Results of analyses of the principal glycolipids of samples of whole saliva and of certain bacteria

The results listed were obtained from analyses performed in duplicate on five individual samples of whole saliva and on two cultures of S. salivarius and of S. mutans.

### TABLE I

<table>
<thead>
<tr>
<th>Chromatographic band*</th>
<th>Reaction with modified benzidine reagent</th>
<th>Susceptibility to mild alkali*</th>
<th>Sugars detected by GLC</th>
<th>Other findings†</th>
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<tr>
<td>Results on whole saliva</td>
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<td>I</td>
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<td>VIII</td>
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<td>G1c:Gal approximately 1:1</td>
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<td>Results on S. salivarius</td>
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<td>DGDG*</td>
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*The chromatographic bands are numbered as shown in Fig. 1A. The chromatographic migration of bands I, V, and VI were markedly affected by treatment with mild alkali. The carbohydrate-containing products of treatment of these bands with mild alkali migrated at or close to the origins of the chromatograms used and were extracted into water. No glycolipids were detected.

† Glycolipid and glucose were detected by GLC as their trimethylsilyl derivatives. The ratios of glycerol and glucose were estimated based on results from similar analyses of standards of mono- and digalactosyl diglycerides. The retention times of glycosylglycerol and diglycosylglycerol were similar to standards of galactosylglycerol and digalactosylglycerol obtained by treatment of mono- and digalactosyl diglycerides with mild alkali. MGDG and DGDG refer to the compounds labeled as such in Fig. 1A.

Results on whole saliva and of certain bacteria.

The major components exhibit migration similar to standards of galactosyl- and lactosylceramides. A small amount of a compound migrating close to the origin of the chromatogram is also evident (indicated by the black dot in the right-hand margin of channel 1). The effect of treatment with mild alkali on the chromatographic migrations of the glycolipids present in the acetone fraction is shown in channel 2. The only glycolipid whose migration was affected by this treatment was the slow migrating compound referred to above. No slower migrating product resulting from treatment of this compound with alkali is evident. However, similar analysis by TLC of the dried water wash obtained subsequent to treatment with mild alkali did reveal the presence of a product migrating very close to the origin of the chromatogram and which gave a positive reaction for carbohydrate when stained with the aniline-diphenylamine reagent (results not shown). The glycolipids present in the acetone:methanol fraction of the same sample of parotid saliva are shown in channel 3 of Fig. 3. Compounds corresponding in migration to standards of galactosylceramide, lactosylceramide, GL-3, and GL-4 are evident. Several glycolipids migrating closer to the origin of the chromatogram are also seen. As shown in channel 4, treatment with mild alkali did not affect the migrations of any of the glycolipids detected in this fraction. Analyses of two other samples of parotid saliva yielded results similar to those shown in Fig. 3. The glycolipids of three individual samples of submandibular saliva were also examined (results not shown). Each of these samples exhibited compounds corresponding in migration to standards of galactosyl- and lactosylceramides as their major glycolipids. Smaller amounts of compounds corresponding in migration to standards of GL-3 and GL-4 were also detected, as were lesser amounts of certain more slowly migrating glycolipids. The submandibular saliva also contained small amounts of an alkali-labile compound that corresponded in migration to the similar compound detected in parotid saliva. None of the other glycolipids in submandibular saliva was affected by treatment with mild alkali.
The glycolipids of human gastric juice—The glycolipids present in five individual samples of human gastric juice were also examined. The amount of glycolipid present in the acetone:methanol fraction from these samples ranged from 2–3 mg/100 ml. Channel 1 of Fig. 4 shows the profile of glycolipids present in the acetone:methanol (9:1, v:v) fraction (prior elution with acetone was omitted) obtained from one sample of pentagastrin-stimulated gastric juice. The principal glycolipids detected corresponded in migration to standards of galactosyl- and lactosylceramides. Treatment with mild alkali (channel 2) affected the migration of only one slow migrating glycolipid (indicated by the black dot in the right-hand margin of channel 1), whereas it markedly affected the migration of sulfatogalactosylalkylacylglycerol (channels 3 and 4). Results identical with those shown in Fig. 4 were obtained by similar analyses of the four other individual samples of gastric juice. No differences in glycolipid profiles were observed between basal and pentagastrin-stimulated samples. The compounds corresponding in migration to galactosyl- and lactosylceramides were isolated by preparative TLC from four of the individual samples of gastric juice and their sugar moieties were analyzed by GLC. Glucose was the principal sugar present in the compound corresponding in migration to the standard of galactosylceramide, although lesser amounts of galactose (approximately 50% of the amount of glucose) were also found in each sample. Analyses of the compound corresponding in migration to lactosylceramide yielded approximately equimolar amounts of glucose and galactose.

Analyses of Glyceryl Ethers—If glyceryl ether-containing glucoglycerolipids are major constituents of either saliva or gastric juice, it would appear reasonable that methanolysis of the glycolipid fraction of these secretions should result in the liberation of appreciable amounts of glyceryl ethers. To investigate this point, portions of the total glycolipid fractions (i.e., obtained by elution with acetone:methanol (9:1, v:v)) of both secretions (from five individual samples of whole saliva and...
five individual samples of gastric juice) were subjected to methanolysis and the products were examined by TLC and GLC. Aliquots of sulfatoxygalactosylalkylacylglycerol, a glycolipid from human whole saliva, were used to study the properties of these compounds. The results of typical analyses by TLC are shown in Fig. 5. Under the conditions of chromatography used, standards of rac 1-octadecylglycerol (betyl alcohol) and of rac 1-octadec-9-enylglycerol (selachyl alcohol) exhibited the same migration as that of 1-hexadecyl-sn-glycerol. Analysis of either the total glycolipid fractions of whole saliva (channel 1, Fig. 5A) or of gastric juice (channel 1, Fig. 5B) revealed the presence of no more than a trace of a compound that exhibited the chromatographic migration of the above three glyceryl ethers. Essentially similar results to these shown in Fig. 5, A and B were obtained from the analyses of the other samples of each of these secretions that were performed. The analyses by TLC of the methanolysates of the total glycolipid fractions of both secretions failed to reveal the presence in either of the compounds corresponding in retention times to the trimethylsilyl derivatives of the three glyceryl ethers just mentioned above. Very substantial peaks corresponding in retention time to the methyl esters of various fatty acids (e.g. C16:0, C18:0, etc.) were evident in these analyses.

![Fig. 5](image-url)

**Fig. 5.** Analyses by TLC of the products of methanolysis of the glycolipid fractions of one sample of whole saliva and of one sample of gastric juice. A: 1, products of methanolysis of the glycolipid fraction of a sample of whole saliva; 2, products of methanolysis of sulfatoxygalactosylalkylacylglycerol; 3, standard of hexadecylglycerol; 4, standard of methyl palmitate. B: 1, products of methanolysis of the glycolipid fraction of a sample of gastric juice; 2, products of methanolysis of sulfatoxygalactosylalkylacylglycerol; 3, standard of hexadecylglycerol. The glycolipid fractions of both secretions were obtained by elution with acetone:methanol (9:1, v:v) (omitting prior elution with acetone). Approximately 0.5 mg of each fraction was used for methanolysis and application to the chromatograms. The amounts of the standards applied to the chromatograms were approximately 20–30 µg. The chromatograms were developed in chloroform:methanol:concentrated ammonia (90:10:0.5, by volume), sprayed with sulfuric acid, and then charred. In the solvent system used, methyl glycosides migrate near the origin of the chromatogram (e.g. the methyl glycosides obtained from sulfatoxygalactosylalkylacylglycerol are seen near to the origin of the chromatogram in channel 2 of A) and long chain bases migrate ahead of these sugar derivatives, in the bottom one-third of the chromatogram. The compound(s) indicated by X have not been characterized, but may correspond to the methyl esters of hydroxy fatty acids.

**DISCUSSION**

These studies have shown that samples of human whole saliva obtained by expectoration contain a complex mixture of glycolipids, the great majority which appear to be glycosphingolipids (cf. Fig. 1 and Table I). The principal glycolipids present in samples of whole saliva corresponded in the properties studied to standards of glucosyl- and lactosylceramides. Most of the glycolipids present in whole saliva were found to be sedimented (Fig. 1B) at a relatively low centrifugal force. This observation, taken along with the findings that both parotid and submandibular saliva contained comparatively low amounts of glycolipids, suggests that the bulk of these compounds was associated with the epithelial and leukocyte cells known to be present in appreciable amounts in whole saliva (25). It should be noted that, although their amounts were relatively small, glucosyl- and lactosyl-ceeramides were also the principal glycolipids present in parotid and submandibular saliva.

Two glucoglycerolipids were detected in the great majority of the samples of whole saliva analyzed. The results of studies designed to partially characterize these latter compounds indicated that they were mono- and diglucosyl diglycerides. Several lines of evidence were adduced to support the view that these two compounds were of bacterial origin. These included the observations that these compounds were not detectable in samples of saliva obtained by cannulation of the ducts of either of the two main salivary glands, that they were sedimented by centrifugation into a pellet that contained not only epithelial cells but also bacteria, and that the amounts of these compounds increased markedly and selectively under conditions of incubation of saliva that favored the growth of bacteria. Moreover, these compounds are known to be the major glycolipids of many bacteria (21, 22) and were found in this study to be the principal glycolipids of *Streptococcus salivarius* and *Streptococcus mutans*, abundant members of the bacterial flora present in the human oral cavity (23). It has been shown previously (26) that mono- and diglucosyl diglycerides are the principal glycolipids of the latter bacterium. Our results indicate that considerable caution should be used in interpreting the significance of detecting glyco glycerolipids in samples of expectorated saliva, in view of the widespread occurrence of bacteria in the oral cavity. It would instead appear to be desirable to perform glycolipid analyses on samples of parotid and submandibular saliva, as these secretions do not normally contain bacteria. When this was done in the present study, no compounds corresponding in chromatographic properties to mono- and diglucosyl diglycerides or to glycerol ether-containing glucoglycerolipids were detected in either of these secretions. Previous investigations of the glycolipids of submandibular (27) and parotid (28) saliva have, however, reported the presence of glyceryl ether-containing glucoglycerolipids in these secretions.

The analyses of human gastric juice performed by us also revealed that compounds corresponding in their properties to standards of glucosyl- and lactosylceramides were the major glycolipids present in that secretion (Fig. 4). No alkali-labile compounds of similar chromatographic migration to the glucosyl glycerides detected in whole saliva were observed in the gastric juice. This finding is perhaps a little surprising, as it might be anticipated that glucoglycerolipids present in whole saliva would pass into the stomach when saliva is swallowed. One possible explanation is that the bacteria in saliva may have become clumped in the oral cavity or by the acid conditions prevailing in the stomach, and that they were removed when the samples of gastric juice were filtered to remove debris (see “Experimental Procedures”) prior to their analysis.
Because of previous reports that the two fluids studied here contained very substantial amounts of glyceryl ether-containing glucoglycerolipids (cf. Ref. 8), special attention was paid by us to detect the presence of these compounds. Particular use was made of the technique of treatment with mild alkali to distinguish between diacyl- and monoalkylmonoacyl-containing glucoglycerolipids (9). Such treatment of the first class of lipid results in the production of water-soluble glyceryl-glycoyl compounds that do not migrate appreciably in non-polar chromatographic solvent systems, as found for the species of mono- and diglucosyl diglycerides detected in this study. In contrast, treatment of the second class of lipid with mild alkali results in lyso derivatives (cf. Fig. 1C) that are relatively insoluble in water. We stress that we did not detect any glycolipids showing both of these latter features during analyses of any of the samples of whole saliva and gastric juice examined here. This observation suggests that monoalkylmonoacyl-containing glucoglycerolipids are not major components of either of these fluids under normal circumstances. In further strong support of this interpretation was the observation that methanolysis of the total glycolipid fractions of these two fluids revealed at most traces of glyceryl ethers (Fig. 5).

At the present time, we are not able to reconcile our findings with those made previously by others (cf. Ref. 8). It is possible that our analyses would have failed to detect the presence of small amounts of glyceryl ether-containing glyceroglycerolipids. This statement applies particularly to compounds of this class containing relatively long oligosaccharide chains (e.g. over four sugars), which would not migrate appreciably from the origins of the chromatoplates in the solvent systems used in this study. We have detected in saliva small amounts of a slow migrating compound (cf. Fig. 1C) that was sensitive to treatment with mild alkali. However, the carbohydrate-containing product resulting from such treatment of this compound was water-soluble. The nature of this compound is at present under study.

REFERENCES

Studies on the Glycolipids of Human Saliva and Gastric Juice

Supplement to: STUDIES ON THE GLYCOLIPIDS OF HUMAN SALIVA AND GASTRIC JUICE
Rajagopalan Narasimhan, Andreas Demick, Burkhard Falter and Robert E. Murray

EXPERIMENTAL PROCEDURES
Collection of samples of saliva and gastric juice.

Samples of whole saliva (approximately 100 ml from each of ten donors) were collected by exocytosis into glass beakers kept on ice. Salivation was induced during this process by chewing pieces of Parafilm (American Can Co., Greenwich, CT 06830). Samples of parotid saliva (approximately 200 ml from each of three donors) were collected by means of Carlson-Crittenden cannula modified by Gurpe (18) and samples of submandibular saliva (approximately 100 ml from each of three donors) were collected with an apparatus described by Trachtenberg (17). The samples were collected into glass bottles containing 0.04 M sodium citrate (pH 6.65) and kept on ice until the time of centrifugation, which was done at 10,000 rpm at 0°C for 30 min. The supernatant and pellet fractions resulting from this procedure were also subjected to lipid extraction. The samples of the pellet fraction obtained by centrifugation of specimens of whole saliva were examined by electron microscopy (kindly performed by Prof. L. Ferszt, Department of Biophysics, University of Toronto).

Isolation of samples of whole saliva.

Samples of approximately 35 ml of saliva were collected by expectation from each of two individuals on two separate occasions, to be used for characterisation of individual glycolipids. On each occasion, the supernatants were discarded and the pellets resuspended in 10 ml of sterile saline solution. The resulting suspensions were centrifuged at just described and the pellets re-suspended in 15 ml of sterile Ringer's solution. A sample of 5 ml of suspension was placed in a dialysis bag and heated at 80°C for 2 h in a water bath. The dialysis bags were subsequently opened and 3 ml of the solution was placed in a separate dialysis bag and dialysed against 10 volumes of 0.2 M NaCl solution for 24 h. The dialyzed solution contained the bulk of the glycolipids and was supplemented with 10 ml of the non-dialyzable gastric lipid extract (15) to give a final concentration of 15 mg/ml of digest. The final lipid extract, containing glycolipids, was incubated with 10 ml of a 2% (w/v) of anhydrous KI solution for 30 min at 37°C. The mixture was centrifuged at 10,000 rpm for 30 min and the supernatant removed. The supernatant was then subjected to thin-layer chromatography (TLC) for the isolation of individual glycolipids. The identity of the individual glycolipids was then confirmed by paper chromatography with the help of authentic standards.

Glycolipids of incubated samples of whole saliva and of certain bacteria.

Because glycolipids have been established to be constituents of various bacteria (29,20) and because bacteria are known to be present in the oral cavity (23), it seemed possible that the glycolipids detected in saliva were derived from bacteria in the oral cavity. Therefore, experiments were performed to test the possibility that bacterial glycolipids might be contributing the glycolipids found in saliva. In order to examine the profiles of glycolipids present in cultures of certain bacteria known to inhabit the oral cavity (15,23), the bacteria selected for examination were Actinomyces naeslundii, Streptococcus salivarius, Streptococcus mutans and Yersinia enterocolitica.

The profiles of the glycolipids extracted from the first two of these bacteria are shown in Fig. 2. The actinomycete (strain 15) is seen to contain small amounts of compound exhibiting the migration of a standard of monosialo-galactosylceramide. The profile of Streptococcus salivarius strain 232 is depicted in Fig. 3. Yersinia enterocolitica strain 12B is shown in Fig. 4. The bacteria selected for examination were Actinomyces naeslundii, Streptococcus salivarius, Streptococcus mutans and Yersinia enterocolitica.

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