Human Salivary Glycosidases[1] (34935)

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The mucosal surface of the stomach is covered by a layer of mucus secreted by the underlying surface epithelial cells and by the prepyloric glands. Approximately 60% of the nondialyzable solids in canine antral mucus consists of large molecular glycoproteins (1). It has been postulated that the latter impart to epithelial mucus its characteristic viscosity which, in turn, appears to be, in part, responsible for the "protective" role postulated for gastric mucus. Although various mechanisms affecting the secretion of mucus by the gastric mucosa have been investigated (2), those which break down the surface coats of mucus are not known. With respect to a possible mucolytic action of gastrointestinal enzymes one must take into consideration the well-known lack of susceptibility of epithelial glycoproteins to degradation by any of the proteases present in gastroduodenal secretions (3). Nor has it been shown that these secretions contain glycosidases capable of digesting protein-bound polysaccharides. However, Leach demonstrated the presence in human saliva of glycosidases capable of degrading protein-bound glycosyl groups of salivary mucins by finding that sialic acid and amino sugars were rapidly released from salivary mucins during incubation of saliva at 37° (4). Since salivary secretions bathe the gastric mucosa continuously, the findings of Leach are relevant to the question raised above concerning the mechanisms responsible for breakdown of the surface coat of mucus covering the gastric mucosa. The purpose of the studies forming the basis of this report was to determine the nature and the sources, bacterial and glandular, of human salivary glycosidases.

Materials and Methods. Salivary collections. Saliva was collected from normal adult men and women laboratory workers and medical students. All subjects had followed good oral hygiene. Collections of whole saliva were achieved by having them chew a small piece of plastic tubing and spit into a sterile flask immersed in ice. Collections lasted for approximately 30 min and yielded from 12–15 ml of saliva. Parotid saliva was collected with a modified Lashley cup. To collect submaxillary–sublingual saliva, a different collector was molded for each subject. For fractional collections, salivary flow was stimulated by applying a cotton applicator soaked with citric acid to the buccal mucosa every 2–3 min. All of the equipment used for fractional salivary collections was gas-sterilized. Cultures of parotid or submaxillary–sublingual saliva collected under these conditions invariably failed to show any growth.

Incubation of saliva. A measured volume of whole saliva was placed in a sterile flask and incubated for up to 24 hr in a Dubnoff shaking water bath. The sample was then exhaustively dialyzed in the cold and lyophilized. The concentration of protein-bound sialic acid, L-fucose, hexoses, and hexosamines per milligram of dry, incubated salivary mucosubstance was estimated according to methods used previously (1). The number of protein-bound glycosyl groups released during incubation was estimated by comparison with the composition of an aliquot of the same sample that had been dialyzed and lyophilized without prior incubation. During extensive preliminary experiments, we had found that this method gave reproducible results with saliva from the same individual,

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Cultivation of salivary samples. The sterility of samples of parotid and submaxillary saliva was determined by plating an aliquot of the collection on blood–agar. Plates were checked for growth after 24 hr of incubation at 37°.

Mixed cultures of the oral flora of individual subjects were prepared in the following manner: an individual sample of whole saliva collected into a sterile flask was incubated at 37° for 6 hr. Two saliva–agar plates prepared by incorporating boiled, human saliva into trypticase–soy agar in the ratio of 1:10 followed by autoclave sterilization, were heavily streaked with the incubated saliva After 24 hr of incubation, the plates were thoroughly washed with sterile broth. The pooled washings from both plates were then added to 200 ml of trypticase–soy broth. After 18 hr of incubation at 37° the broth was centrifuged at 30,000*g* for 30 min in a Sorval refrigerated centrifuge. An aliquot was carefully removed from the top layer of the supernatant fraction and was assayed for extracellular glycosidases. The sediment was resuspended in 15 ml of cold, sterile isotonic saline and was sonified in the cold for 10 min; cellular debris was removed by centrifugation at 30,000*g* for 30 min. The concentration of intracellular glycosidases in the supernatant fraction was then assayed.

Assays for glycosidase activities. Neuraminidase (*N*-acetyl-neuraminate glycohydrolase, EC 3.2.118) was assayed by incubating 0.2 ml of whole saliva, parotid, or submaxillary–sublingual saliva, or culture broth with 0.1 ml of a solution containing 5 mg/ml of *N*-acetylneuraminyllactose and 0.5 ml of 0.1 *M* phosphate–citrate buffer, pH, 6.3, for 60 min at 37°. The reaction was terminated by the addition of Na metaperiodate reagent and the released free sialic acid in the mixture was estimated (1). Neuraminidase activity in the sample of saliva was expressed as micromoles per liter-minute of sialic acid released from the substrate by 1 ml of saliva under the conditions of the assay.

β-D-Galactosidase (β-D-galactoside glycohydrolase, EC 3.21.23) was assayed by incubating 0.4 ml of saliva or broth with 0.1 ml of a 10 m*M* solution of *o*-nitrophenyl β-D-galactopyranoside and 0.5 ml of 0.1 *M* phosphate–citrate buffer, pH 6.3, for 60 min at 37°. The reaction was terminated by the addition of 1.0 ml of 0.25 *M* Na_2CO_3, and the developed yellow color was measured immediately at 430 mμ. The β-D-galactosidase in the saliva or broth was expressed as micromoles per liter-minute of phenolic aglycon released from the substrate per 1 ml of saliva or broth under the conditions of the assay.

β-*N*-Acetylglucosaminidase (β-2-acetamido-2-deoxy-D-glucoside-acetamidodeoxyglucohydrolase, EC 3.2.1.30), α-glucosidase (α-D-glucoside glucohydrolase EC 3.2.1.20), β-glucosidase (β-D-glucoside glucohydrolase EC 3.2.1.21), α-galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22), α-mannosidase (α-D-mannoside mannohydrolase EC 3.2.1.31) were assayed similarly by incubating saliva or broth with phenolic glycoside substrates and 0.1 *M* phosphate–citrate buffer of appropriate pH. Because of the poor solubility of the fucoside, 0.5 ml of a 2 m*M* solution of substrate was used for the assay of α-L-fucosidase (α-L-6-deoxygalactoside-deoxygalactohydrolase). The pH optima for the various enzyme systems assayed are summarized in Table II. To ensure that the enzyme assays were done under conditions of zero-order kinetics, incubation times were varied from 0–60 min in preliminary experiments. During routine assays, for which an incubation time of 60 min was used, the degree of hydrolysis of substrate never exceeded 15% and was usually less than 10%. Parallel substrate and enzyme blanks were incubated under identical conditions and extinction values thus obtained were subtracted from those obtained from the corresponding experimental digestion mixtures.

The coefficients of variation of 10 replicate assays of one sample of saliva from one subject for neuraminidase, β-D-galactosidase, α-L-fucosidase, *N*-acetyl-β-D-glucosaminidase, and α-D-mannosidase were 3.06%, 1.9%, 1.35%, 0.38%, and 3.4%, respectively. Also, there was a linear relationship between the rate of substrate degradation and the amount of enzyme (volume of saliva or broth) in the incubation mixture for all enzymes studied.

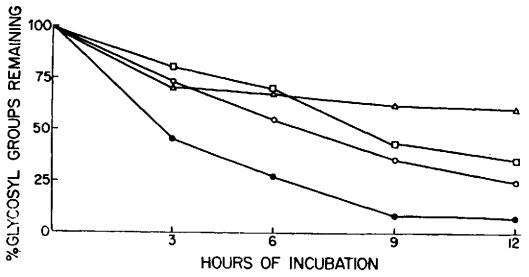


FIG. 1. Release of protein-bound carbohydrates of salivary mucins during incubation of saliva. Measured aliquots of whole saliva were incubated for 3, 6, 9, and 12 hr and were then dialyzed and lyophilized. A measured aliquot from the same sample was dialyzed and lyophilized without prior incubation. From analysis of concentration of protein-bound carbohydrates in the various aliquots, the amount of each glycosyl group released (dialyzable) during incubation was estimated. ●—●: sialic acid; ○—○: L-fucose; □—□: hexosamines; △—△: hexoses.

Results. Incubation of saliva. Salivas of 15 subjects were incubated for 24 hr as described above. After 24 hr of incubation, the average loss of protein-bound carbohydrate from salivary mucosubstance was 58%. For the individual glycosyl groups, the losses were as follows: sialic acid (82%); hexosamines (56%); hexoses (54%); L-fucose (68%). A representative experiment is illustrated in Fig. 1. In preliminary experiments, we found, as did Leach (4), that release of protein-bound carbohydrate during incubation of saliva does not occur when the saliva has been boiled previously. We also found that there was no release of protein-bound carbohydrate during incubation of sterile submaxillary–sublingual saliva.

Assays of salivary glycosidases. Separate samples of whole, parotid, and submaxillary–sublingual saliva were collected from 18 adult subjects and were assayed for neuraminidase, β-D-galactosidase, β-*N*-acetylglucosaminidase, and α-L-fucosidase activities. The results are summarized in Table I. The whole saliva of all 18 subjects contained α-L-fucosidase and β-*N*-acetylglucosaminidase. β-D-Galactosidase and neuraminidase activities were not detectable under the conditions of the assay in two and eight of the subjects, respectively. In the salivas of the other subjects, the activities of these two enzymes followed each other closely. In the parotid and submaxillary–sublingual salivas of all 18 subjects, α-L-fucosidase and β-*N*-acetylglucosaminidase were invariably present; however, neuraminidase and β-D-galactosidase activities were never detected. The data suggest that the α-L-fucosidase and β-*N*-acetylglucosaminidase activities in whole saliva originate in part in the salivary glands. In 5 of the 18 subjects, whole saliva was also assayed for α-galactosidase, and α-and β-glucosidase, β-glucuronidase, and α-mannosidase. With the exception of β-glucosidase, which was found in the whole saliva of only one of the subjects, these enzymes were invariably present in the whole saliva (Table II). In none of the five subjects did sterile parotid or submaxillary saliva contain any of these enzymes. pH activity curves of submaxillary–sublingual α-L-fucosidase and β-*N*-acetylglucosaminidase were determined with salivas from the five subjects. The results are shown in Fig. 2. The pH optima for the two enzymes were found to be 5.5 and 4.5, respectively.

Individual mixed cultures of the oral flora of five subjects contained all of the glycosidases listed in Table II, except α-manno-

TABLE I. Exoglycosidases in Saliva of 18 Normal Human Subjects.[a]

	Whole saliva	Parotid saliva	Submaxillary–sublingual saliva
Neuraminidase	2.75 ± 2.92	0	0
β-D-Galactosidase	0.28 ± 0.26	0	0
β-*N*-Acetyl-D-glucosaminidase	9.58 ± 3.10	5.07 ± 2.93	4.32 ± 2.57
α-L-Fucosidase	0.52 ± 0.22	0.23 ± 0.17	0.28 ± 0.23

[a] All values listed = average ± SD velocity expressed as micromoles per liter-minute of substrate digested per milliliter of saliva.

sidase. β-Glucosidase was present in the mixed broth culture of only one subject and was later recovered from a pure yeast culture from the saliva of the same subject. Probably β-glucosidase would have been recovered more often had the cultures been grown under conditions favorable to the growth of yeast. β-Glucuronidase and α-L-fucosidase activities were found only after sonication of the organisms which suggests that both of these enzymes are intracellular in so far as the organisms from the five subjects in the study are concerned. The pH activities of the intracellular and extracellular glycosidases in mixed cultures are summarized graphically in Fig. 2. It will be seen that whereas submaxillary–sublingual α-L-fucosidase and β-glucosaminidase from five subjects had pH optima of 5.5 and 4.5, bacterial α-L-fucosidase and β-glucosaminidase in mixed cultures from the same five subjects had pH optima of 6.0 and 5.5.

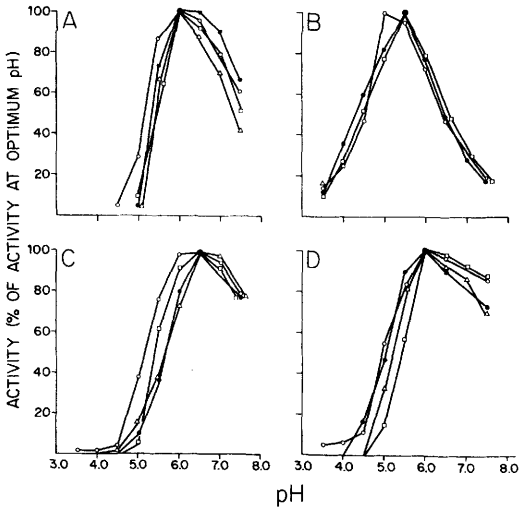
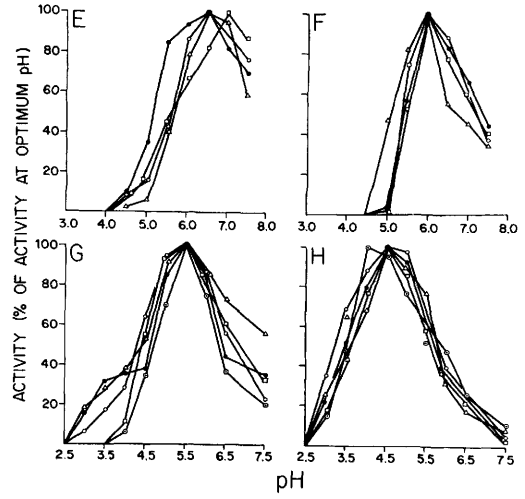


FIG. 2. pH activity curves of α-glucosidase (A), β-*N*-acetylglucosaminidase (B), α-D-galactosidase (C), β-D-galactosidase (D), neuraminidase (E), and α-L-fucosidase (F) from mixed broth cultures of oral flora from five human subjects. G and H represent pH activity curves of α-L-fucosidase and β-*N*-acetylglucosaminidase in submaxillary–sublingual saliva from the same five subjects. The values are plotted as percentages of activity at optimum pH.



The source of salivary α-D-mannosidase remained undetermined. This enzyme was present in the whole saliva of five subjects with, in all instances, appreciable activity and a common peak of optimum pH activity (4.0). In no instance was mannosidase activity detected in broth cultures (or in the sonicated sediment) of mixed oral flora from the same five subjects. α-D-Mannosidase activity was not found in parotid or submaxillary–sublingual salivas from the same five individuals despite the addition of Zn^{2+} and .075% bovine serum albumin to the incubation mixtures. Furthermore, the α-D-mannosidase activity in the salivas of 12 individuals did not increase during 24-hr incubation at 37° of these salivas (Table III). By contrast, the activity of all the other enzymes identified with a bacterial origin increased markedly during incubation of saliva. The data suggest that salivary α-D-mannosidase does not come from the oral microflora but may originate from buccal glands or cellular material (leukocytes?) in saliva.

Discussion. In studies such as this in which synthetic substrates are used, one must not lose sight of the fact that the ability to hydrolyze a specific linkage in a synthetic glycoside does not prove that the same enzyme system can attack the same linkage in a naturally occurring macromolecular substrate. However, this objection is less critical in this particular study since it is obvious (Fig. 1) that the glycosidases in human saliva are capable of degrading salivary mucins.

TABLE II. Sources of Exoglycosidases in Human Saliva.

	α-D-Galactosidase	β-D-Galactosidase	α-D-Glucosidase	β-D-Glucosidase[a]	β-*N*-Acetylglucosaminidase	α-L-Fucosidase	β-D-Glucuronidase	α-D-Mannosidase	Neuraminidase
Whole saliva	+	+	+	±	+	+	+	+ 4.0	+
Parotid saliva	0	0	0	0	+ 4.5	+ 5.5	0	0	0
Submaxillary–sublingual saliva	0	0	0	0	+ 4.5	+ 5.5	0	0	0
Mixed culture of oral flora	+ 6.5	+ 6.0	+ 6.0	± 8.5	+ 5.5	+[b] 6.0	+[b] 7.0	0	+ 6.5

[a] β-Glucosidase present in only one of five subjects; values listed = pH optima.
[b] Activity present only after sonication of cultures.

In this study, we were particularly interested in determining the presence in saliva of the four enzymes: neuraminidase, α-L-fucosidase, β-D-galactosidase, and β-*N*-acetylglucosaminidase since structural studies of various glycoproteins have shown that the carbohydrate sequence–sialic acid (or fucose) → galactose → *N*-acetylglucosamine—is a preferred one. All of the enzyme systems which one would expect to find in saliva from the results of the studies with incubated saliva can be accounted for on the basis of assays with artificial substrates.

Our data show that human saliva contains at least two different α-L-fucosidases: one secreted by the parotid and submaxillary-sublingual glands and the other produced by oral bacteria. Each one of these enzyme systems has a different pH of optimum activity.

TABLE III. Influence of Incubation on Activity of Salivary Exoglycosidases.[a]

	Time	
	0	24 hr
β-D-Galactosidase (12)	.68 ± .41	3.89 ± 1.83
α-D-Mannosidase (10)	.44 ± .16	.42 ± .15

[a] Values listed = av ± SD velocity expressed as micromoles per liter-minute of substrate digested per milliliter of saliva; 0 time = assay of fresh saliva; 24 hr = assay after 24 hr of incubation of saliva at 37°; ( ) = number of individual salivary samples assayed; increase in activity of salivary β-D-galactosidase with incubation of saliva is representative of what was found with all enzymes identified with bacterial origin.

The same is true of β-*N*-acetylglucosaminidase.

Neuraminidase plays a particularly important role in the enzymatic degradation of sialic acid-containing glycoproteins. Because sialic acid always occupies a terminal position on the oligosaccharide prosthetic groups of glycoproteins, release of other glycosyl groups by other glycosidases cannot occur until the terminal sialyl groups have been removed by neuraminidase. Moreover, as the sialyl groups are released, the polypeptide core of the glycoprotein molecule becomes susceptible to attack by endopeptidases (3). Our data show that neuraminidase is not secreted by the human salivary glands. Although Perlitsh (5) reported finding neuraminidase activity in human submaxillary–sublingual saliva, his assay method was susceptible to bacterial contamination. Our observations are in agreement with those of Rølla (6) and of Nijjar, *et al.* (7), who concluded that salivary neuraminidase originates only from oral bacteria.

As far as determining the specific organisms responsible for the production of each of the bacterial enzyme systems is concerned, we have isolated from the five subjects whose saliva we studied two different α-hemolytic streptococci, the one (var mitis) producing neuraminidase, β-D-galactosidase and β-*N*-acetylglucosaminidase, and the other producing α-D-galactosidase and α-D-glucosidase. Others have described identical enzyme production by oral streptococci (7).

It seems clear that human saliva contains glycosidases capable of degrading epithelial mucins. Most of the activity originates from oral bacteria. Of particular significance, is the finding that neuraminidase, a key enzyme in the degradation of epithelial mucins, is present only in bacteria-containing saliva. The enzyme systems which we have assayed in saliva all have acid pH optima which, given certain conditions, would enable them to attack the mucous lining of the stomach. These conditions would be a reduced level of acid secretion by the stomach and gastric stasis, both of which may occur in patients who, for one reason or another, are in shock. Bacteria have been found in the gastric mucosa of patients developing stress ulcers after severe thermal burns (8). It is conceivable that when conditions prevailing in the stomach are favorable to the growth of oral organisms, the glycosidases produced by the latter may break down the surface mucus of the stomach more rapidly than it can be renewed by the surface epithelium. Such a weakening of the mucosal barrier might explain the multiple gastric erosions often complicating severe "stress" in humans.

Summary. The activity of several glycosidases—neuraminidase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-*N*-acetylglucosaminidase, α-mannosidase, β-glucuronidase, and α-L-fucosidase—was assayed in whole, parotid, and submaxillary–sublingual human saliva as well as in mixed cultures of oral microflora. All of these enzymes were present in whole saliva and, with the exception of α-mannosidase, were also present in mixed broth cultures of oral organisms from the same subjects. Only α-L-fucosidase and β-*N*-acetylglucosaminidase were found in sterile parotid and submaxillary–sublingual saliva. The latter had peaks of optimal pH activity that differed from those of the corresponding bacterial enzymes recovered from the same subject. Since salivary glycosidases can degrade salivary mucins they may be capable, under certain conditions, of attacking the surface layer of mucus in the stomach.

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