Characterization of Whole, Canine Gastric-Antral Mucus[1] (34306)

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The apparent invulnerability of the gastric mucosa to the high acidity of its contents has long intrigued physiologists. In 1954 Hollander (1) formulated the concept of a protective, 2-component mucosal barrier consisting of the surface epithelium covered by a layer of adherent mucus, concept upon which subsequent investigations of the role of mucus in the health and disease of the gastric mucosa were based. The following quote from Turner *et al.* (2) is a pertinent commentary on the validity of such studies: "The studies on gastric mucus are still bedeviled by a lack of adequate characterization of components of this mixture." It is obvious that further attempts to clarify the protective role of mucus are not likely to be fruitful until more is known about the nature of this secretion. Our attempts to characterize *whole, fresh* gastric mucus form the basis for this report. *Whole* mucus indicates that we studied mucus in its native state as it is secreted by the pyloric glands into the lumen of the antrum. *Fresh* indicates that we studied material that had not been previously denatured by physical or chemical agencies.

Materials and Methods. Collection of mucus. Vagally denervated pouches were constructed from the gastric antrum of 4 dogs. Care was taken to exclude acid secreting mucosa from the pouches. In 3 dogs the pyloric end of the pouch was sewn to the skin of the stab-wound in the anterior wall (antrostomy). In the fourth dog, the influence of a cannula on the secretion of mucus was studied by draining the pouch secretions via a small, flanged plastic cannula. Collections were started after a 6-week recovery period. Collections were made for periods of 5–6 hr in the morning after a fast of approximately 20 hr. After a suitable number of collections from the dog with the cannula, the latter was removed and a cutaneous antrostomy was constructed with the pyloric end of the pouch, as in the 3 other animals. Collections were then resumed after 6 weeks.

Polyacrilamide-Gel Column Chromatography. For this part of the study, each daily, individual collection of fresh mucus from 2 dogs was submitted to the following procedures. A measured aliquot of mucus was placed in a Visking dialysis bag and dialyzed at 4° for 48 hr against multiple changes of distilled H_2O and then lyophilized. By careful weighing of the dry material an estimate of the concentration of nondialyzable mucosubstance in the sample of fresh mucus was obtained. Another aliquot of the same sample of fresh mucus was diluted (3:17; v/v) with Sorensen's phosphate buffer (pH 7.5) containing 0.1% NaN_3, homogenized and centrifuged at 200*g* for 10 min. The supernatant was carefully decanted. The mucoid precipitate was dialyzed and lyophilized and weighed as above. Two 5.0-ml aliquots of the supernatant were then applied to each of two 2 × 22-cm P-300[3] polyacrilamide columns previously equilibrated with the same phosphate buffer. The sample was passed through the column at a rate of approximately 10 ml/hr. Fractions of 1.5 ml were collected and the elution was monitored by the measurement of the absorbance at 280 mμ. Contents of the tubes under the peaks were pooled, dialyzed for 48 hr at 4°, lyophilized, and weighed. From the amount of non-

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dialyzable mucosubstance in the precipitate and in the column fractions recovered from each individual daily sample of mucus, the relative proportions of each fraction in whole mucus were estimated. (The precipitate was considered to be a fraction of whole mucus.) A total of 9 daily collections from each of 2 dogs were thus processed. For each dog, the whole mucus, precipitate, and column fractions were pooled separately for subsequent biochemical analysis.

Analytical Procedures. The method of Lowry (3) was used for the determination of protein concentration in whole, fresh mucus. The concentration of nitrogen in lyophilized mucosubstance was measured by the micro-Dumas method using an automated instrument.[4] Protein concentration was calculated from the measured nitrogen concentration (minus the sialic acid and hexosamine nitrogen) by using 6.25 as the correction factor. Neutral and amino sugars were liberated by hydrolyzing 6–7 mg aliquots of material in sealed, evacuated tubes at 100° for 2 hr in 3 ml of 2 *N* HCl. The hydrolyzates were dried *in vacuo*. Hexosamines were measured by the method of Dische and Borenfreund, using a 25-μg standard (4). Hexoses and L-fucose were measured by the method of Dische and Shettles (5) using a standard consisting of galactose, mannose, and L-fucose in amounts of 25, 25, and 10 μg/ml, respectively. To determine the concentration of sialic acid, 3–4 mg of lyophilized material were hydrolyzed at 80° for 1 hr in 1 ml of 0.1 *N* H_2SO_4. The released sialic acid was measured by the thiobarbituric method of Warren (6). Uronic acids were liberated by hydrolysis of 10 mg of material at 100° for 10 min and were analyzed by a modification of the carbazole method of Bitter and Muir (7) permitting the inclusion of a blank for the remainder of the analytical procedure.

Electrophoresis. Acrylamide gel disc electrophoresis of whole mucus from 4 dogs was carried out according to the method of Davis (8). Whole mucus was electrophoresed by treating it as a dilute protein solution and incorporating 56 μl of material in the sample gel. Mucus from the dog with an antral cannula had a higher protein concentration and required dilution before its incorporation in the sample gel. The gels were stained with amido black and scanned with a model F[5] microdensitometer. The concentration of protein in the starting material was estimated by the method of Lowry (3). When appropriate, an alternate gel from the same sample was stained with PAS.

Electrophoretic characterization of the fractions recovered from mucus chromatographed on polyacrilamide columns was carried out as above after concentration of the eluted fractions with G-100 Sephadex.

Immunoelectrophoresis. Electrophoresis on cellulose acetate membranes in Veronal buffer (ionic strength: 0.075; pH 8.6) was carried out with the Beckman microzone cell. Whole serum and whole mucus or concentrated column fractions were applied simultaneously. Upon completion of the electrophoresis 20 μl of rabbit antiserum to whole canine serum were applied to troughs in the membrane. The precipitin arcs were stained with Ponceau S.

Results. Fractionation of whole, fresh mucus. The processing of fresh, whole mucus as described above led to its separation into four crude fractions:

Fraction I: An insoluble precipitate forming when mucus was diluted with phosphate buffer.

When the dilute supernatant was applied to a polyacrilamide column, three other fractions were separated:

Fraction II: This fraction was eluted in the void volume of the column (mol wt >300,000).

Fraction III: This fraction consisted of material eluted under the second chromatographic peak.

Fraction IV: This fraction consisted of material eluted under the third chromatographic peak.

The total weight of dry material recovered in each fraction from every run gave an estimate of the contribution of each fraction to the overall composition of the nondialyzable

[4] Coleman nitrogen analyzer.

[5] Canalco.

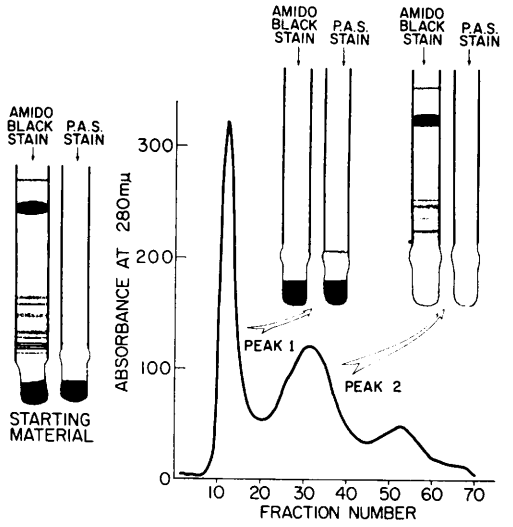

FIG. 1. Typical gel filtration chromatogram of fresh, *soluble* (after separation of insoluble Fraction I) mucus on a P-300 column. Disc-gel electrophoretic patterns of starting material and of material from column peaks 1 and 2 are illustrated.

mucosubstance. Fraction I represented 17 and 24% of the recovered material in dogs DP-45 and DP-46, respectively. Corresponding values for Fractions II, III, and IV were: 44 and 33%; 31 and 34%; 6 and 7%. As pointed out below, Fractions I and II consist primarily of glycoproteins, and Fractions III and IV consist mainly of serum proteins (III) and other materials of lesser molecular weight (IV). The data indicate that prepyloric mucus consists of a "mucoid" portion and a protein portion, which represent approximately 60 and 40%, respectively, of the nondialyzable solids in mucus.

The Sephadex column fractions were further characterized by disc electrophoresis in addition to biochemical analysis as described below. Fraction I, being insoluble, did not lend itself to electrophoretic analysis. As shown in Fig. 1, Fraction II (material eluted in the void volume (peak 1) of the column) consisted almost entirely of material remaining in the sample gel and highly positive for both amido black and PAS stains. This fraction was slightly contaminated by one or sometimes two faintly PAS positive bands with weak anodal mobility. Fraction III (second chromatographic peak) consisted of

TABLE I. Composition of Whole Mucus and Fractions of Whole Mucus from the Gastric Antrum of 2 Dogs.

		% of all material recovered[a]	Protein[b]	Sialic acid[b]	Hexosamines[b]	Hexoses[b]	L-Fucose[b]	Uronic acids[b]	Acetyl groups[b]	Total carbohydrate[b]	Carbohydrate/protein	% Analytical recovery
Whole mucus	DP 45	—	594.9	10.7	96.5	85.9	42.5	5.2	22.6	263.4	0.4427	85.8
	DP 46	—	606.0	9.4	122.8	78.2	46.8	4.8	28.9	290.8	0.4798	89.7
Fraction I (insoluble)	DP 45	17.41 ± 3.49	524.7	7.8	68.9	82.8	34.0	7.4	16.2	217.1	0.4137	74.2
	PP 46	24.33 ± 4.83	511.4	9.2	132.3	92.3	46.9	9.3	31.0	321.0	0.6276	83.2
Fraction II	DP 45	44.65 ± 3.40	598.0	11.6	104.5	112.6	48.8	7.4	24.5	309.4	0.5173	90.7
	PP 46	33.64 ± 4.25	504.6	8.6	99.6	91.3	45.8	8.8	23.4	277.5	0.5499	78.2
Fraction III	DP 45	31.59 ± 9.32	926.1	2.8	14.0	30.3	6.2	6.7	3.3	63.3	0.0680	98.9
	DP 46	34.35 ± 6.87	872.9	1.8	13.9	26.2	6.4	6.6	3.3	58.2	0.0666	93.1
Fraction IV[c]	DP 45	6.33 ± 2.58	—	—	—	—	—	—	—	—	—	—
	DP 46	7.67 ± 2.68	—	—	—	—	—	—	—	—	—	—

[a] Average of 9 fractionations in each of 2 dogs.
[b] Freeze-dried material (μg/mg) after correction for moisture and ash.
[c] Material in Fraction IV was insufficient amount for biochemical analysis.

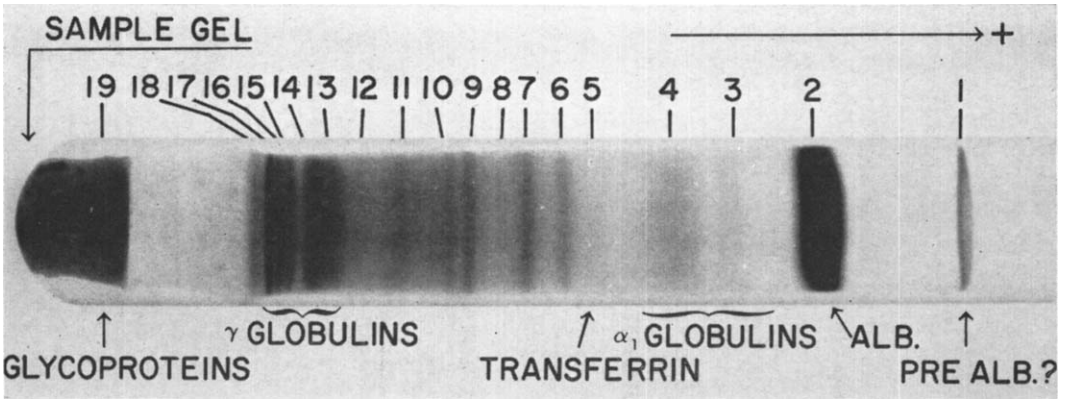

FIG. 2. Acrylamide gel disc electrophoresis of *fresh, whole,* canine gastric antral mucus; amido black stain.

multiple amido-black-positive fractions with the mobility and immunoelectrophoretic characteristics of serum proteins.

Electrophoretic analysis of Fraction IV revealed only a very faint anodal band with a mobility idential to that of serum albumin.

Analytical Procedures: These are summarized in Table I. Except for minor differences in concentrations of hexosamines and hexoses, the protein and carbohydrate composition of whole, fresh mucus from the two dogs were similar. Fractions I and II both had a high carbohydrate concentration with a high carbohydrate-to-protein ratio and appear to consist primarily of glycoproteins. On the basis of this relatively small sampling, it was not possible to find any consistent differences between Fractions I and II. In one dog, the carbohydrate-to-protein ratio was substantially lower in Fraction I than in II. However, the reverse was found in the other dog. Fraction III, as already indicated by electrophoretic analysis, had a very low concentration of protein-bound carbohydrate and a low carbohydrate-to-protein ratio: 0.07 for both dogs.

Since only very small amounts of material from Fraction IV were recovered, it was possible to carry out only fragmentary analyses. The latter suggest a very low concentration of bound carbohydrate. In all likelihood, this fraction consists primarily of small molecular weight peptides and some albumin spilling over from Fraction III.

Electrophoresis and immunoelectrophoresis. Disc electrophoresis of fresh mucus revealed a pattern common to all dogs and consisting of at least 19 fractions (Fig. 2). A large amount of material remained in the sample gel. This material and one or two faint bands with very weak anodal mobility were the only fractions positve for PAS. The heavy anodal band was shown to be albumin by simultaneous electrophoresis of canine serum and by immunoelectrophoresis. Only tentative identification was made of the other anodal-moving proteins. The relative amounts of protein present in whole, fresh mucus as albumin, nonalbumin mobile proteins (globulins), and nonmobile carbohydrate-bound protein were determined by densitometric analysis of the gels and are given in Table II. In three dogs (antrostomy) the average amount of protein bound to carbohydrate (glycoprotein) represented 30% of the total protein in the sample of mucus measured by the method of Lowry.

Immunoelectrophoresis of fresh, whole mucus and of canine serum on cellulose-acetate membranes showed that the immunoelectrophoretic pattern of whole mucus was similar to that of serum when both were reacted against rabbit antiserum to whole canine serum (Fig. 3). An identical pattern to that shown in Fig. 3 obtained with whole mucus was found upon immunoelectrophoresis of Fraction III (column peak 2). Using partially purified rabbit antisera against canine serum protein fractions, the following canine serum proteins in whole mucus were tenta-

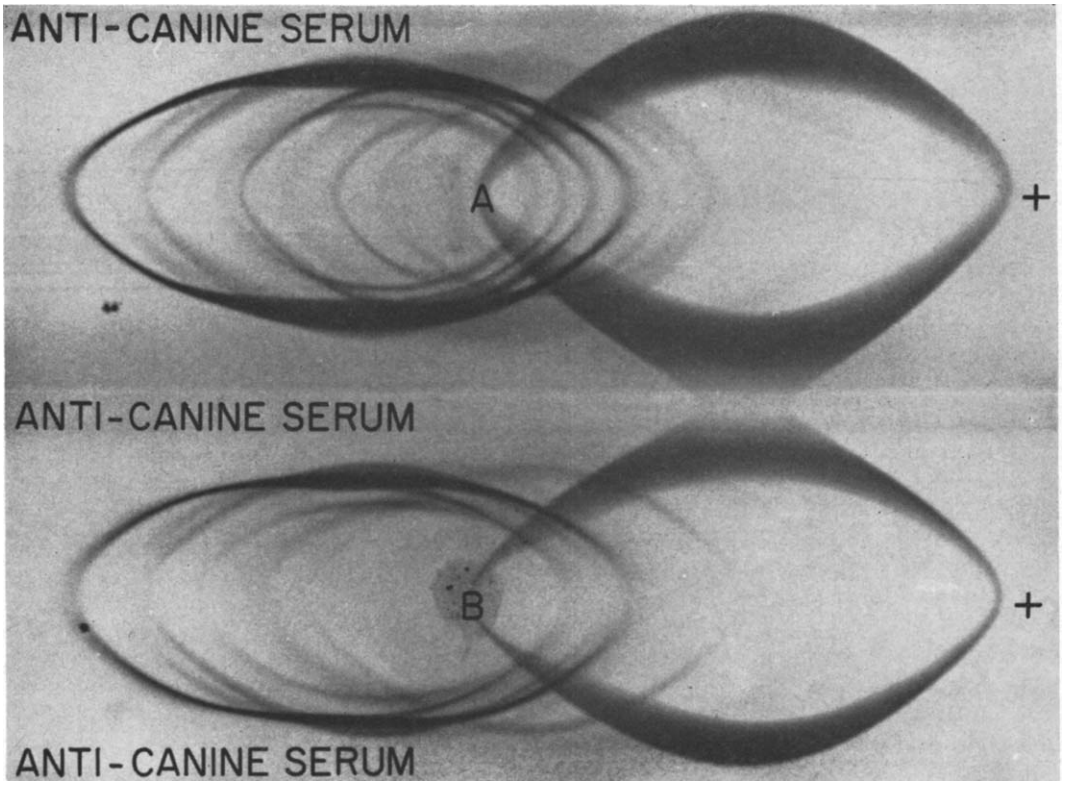

FIG. 3. Immunoelectrophoresis of serum (A) and whole, fresh gastric-antral mucus (B) from the same dog. Rabbit antiserum to whole canine serum was applied to the 3 troughs. Precipitin arcs stained with Ponceau-S.

TABLE II. Acrylamide Gel Disc Electrophoresis of Proteins in Fresh, Whole Canine Antral Mucus.[a]

Dog	Total protein[b]	Albumin	Nonalbumin mobile proteins "globulins"	Nonmobile carbohydrate bound protein
DP-45	0.518 ± 0.071	0.130 ± 0.033 (25%)	0.236 ± 0.044 (45%)	0.151 ± 0.024 (29%)
DP-46	0.406 ± 0.099	0.101 ± 0.028 (25%)	0.157 ± 0.040 (38%)	0.147 ± 0.042 (36%)
DP-47	0.447 ± 0.168	0.102 ± 0.032 (25%)	0.222 ± 0.094 (50%)	0.120 ± 0.047 (27%)
DP-44(a)	2.292 ± 0.814	0.465 ± 0.193 (20%)	1.154 ± 0.399 (50%)	0.671 ± 0.295 (29%)
(b)	0.322	0.090 (22%)	0.197 (47%)	0.130 (31%)

[a] All values represent mean g/100 ml ± SD of 7 electrophoreses of 7 different samples from each animal. [DP-44(b) = mean of 2 electrophoreses of 2 different samples]; (%) = % of total protein. DP-44(a) collections with cannula in antrum; DP-44(b) collections without cannula in antrum.

[b] Measured by the method of Lowry.

TABLE III. The Influence of an Indwelling Antral Cannula on the Composition of Gastric Mucus.

	Sialic acid[a]	Hexosamines[a]	Hexoses[a]	Fucose[a]	Total carbohydrate	Protein[a]	Carbohydrate /protein	% Analytical recovery
With cannula in antrum (1)[b]	4.1	19.0	26.1	5.6	54.8	768.7	0.071	82.4
After removal of cannula (7)	11.2 ± 2.2	106.7 ± 15.8	74.2 ± 9.0	34.7 ± 4.1	226.7 ± 25.6	599.0 ± 47.9	0.381 ± 0.064	83.0

[a] Freeze-dried, dialyzed whole mucus (μg/mg).
[b] Number of collections analyzed given in parentheses.

tively identified: albumin, alpha-1 globulins, gamma globulins, transferrin. A more careful identification of the serum proteins in gastric mucus was not attempted in the scope of this study.

Influence of an antral cannula on the composition of gastric mucus. The influence of an indwelling antral cannula on the protein and carbohydrate composition of canine antral mucus is summarized in Tables II and III. With the cannula in place the mucous secretions from the antrum of this one dog were characterized by a high protein concentration. However, the relative amounts of protein present in the mucus as albumin, nonalbumin, mobile proteins and nonmobile carbohydrate-bound protein were similar to those found in the dogs with an antrostomy and did not change appreciably after removal of the cannula. After removal of the cannula there was a net decrease in protein concentration which was exactly compensated for by a net gain in the concentration of protein-bound carbohydrate. The latter increased to a value comparable to that found in dogs DP-45 and DP-46 with an antrostomy.

Discussion. It is evident from this study that the relative proportions of protein and protein-bound carbohydrate in antral mucus may be influenced considerably by the presence of an indwelling cannula. It is probable that the cannula traumatizes the antral mucosa and increases the rate of secretion of mucus and alters the composition of gastric mucus. On the other hand, the protein and protein-bound carbohydrate concentrations have been quite constant (in this study as well as in other studies in progress) from one dog to another in the mucus collected from antrostomy pouches (without a cannula). These data suggest that in future studies of canine gastric-antral mucus the use of an indwelling cannula should be avoided.

Our studies indicate that the gross characteristics of gastric-antral mucus are similar to those of other mucous secretions (salivary, cervical): a mixture of large molecular weight glycoproteins and proteins immunologically idential to serum proteins. The glycoprotein or mucoid fraction of gastric mucus is obviously heterogeneous. At the very least it

consists of a soluble and an insoluble fraction in phosphate buffer. The similar formation of a "mucin clot" is known to occur with saliva. It is possible that the insoluble material results from some rapid alteration (enzymatic? bacterial?) of the original material. As far as the soluble glycoprotein in material in Fraction II is concerned, we have been unable to subfractionate it into different molecular species on the basis of molecular weight using agarose columns capable of excluding material with a mol wt $> 1.5 \times 10^6$. It is, of course, notoriously difficult to apply such chromatographic techniques to the separation of native, epithelial glycoproteins because of the extraordinary tendency of these secretions to adhere tenaciously to all chromatographic supporting media.

The demonstration of serum proteins in canine gastric mucus is not a new observation. Horowitz and Hollander (9) found that mucus from canine Heidenhain pouches contained albumin and alpha, beta, and gamma globulins. However, the samples of mucus from our antral pouches contained all, or almost all, of the serum protein fractions as shown by disc electrophoresis and immune reaction of fresh mucus against antiserum to canine whole serum. It is evident that even a brief contact with parietal cell secretions suffices to denature the serum proteins present in mucus, thus rendering their identification impossible. In experiments not described in this paper, we found that the normal disc-electrophoretic pattern of fresh antral mucus (Fig. 2) was profoundly altered by brief exposure of the mucus to acid gastric juice. Similarly, lyophilized canine gastric-antral mucus revealed, when redissolved in buffer and electrophoresed, only a few weakly staining protein bands. Our observations suggest that the more detailed identification and quantitation of serum proteins in gastric mucus which remains to be done should be carried out on *fresh antral* mucus. At this juncture one can only speculate on the biologic role of serum proteins in gastric mucus. It is possible that certain components, such as the gamma globulins, may participate in immune defense mechanisms in the alimentary tract and thus help to control the bacterial flora of the gut. Albumin may enhance the solubility of the glycoprotein components of mucus or retard denaturation of enzyme-protein. As far as the mucoid portion (Fractions I and II) is concerned, it is clear that the glycoprotein nature of the material renders it quite resistant to proteolysis. We found that, under optimum conditions for peptic and tryptic activity, digestion of the protein in these two fractions by these enzymes over a period of 3 hours was virtually nil. This characteristic should retard the rate at which the surface layer of mucus is broken down and wiped off by peristalsis. However, beyond this, the exact mechanism by which this layer of mucus "protects" the underlying epithelium, if indeed it does have such a protective function, remains to be elucidated.

Summary. Fresh mucus, collected from the canine gastric antrum, was studied by column chromatography, disc gel and immunoelectrophoresis, as well as by analytical techniques for the measurement of protein and protein-bound carbohydrate concentration in mucosubstance. Approximately 40% of the nondialyzable organic solids in fresh antral mucus consists of serum proteins with representation of all or almost all of the various serum protein fractions. The rate of mucous secretion is augmented and the relative proportions of protein and protein-bound carbohydrate are altered when an indwelling cannula is used for the collection of antral mucus.

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