

HISTOCHEMICAL STAINING AND CHARACTERIZATION OF GLYCOPROTEINS IN ACID-SECRETING CELLS OF FROG STOMACH

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ABSTRACT

Glycoproteins were histochemically localized in oxyntic cells of the frog stomach by staining with periodic acid-silver methenamine. Reduction of silver was most intense on (a) the outer aspect of the apical plasmalemma, (b) within the tubular smooth membrane system characteristic of oxyntic cells, and (c) within cisternae and vesicles of the Golgi complex. Other membrane components such as those from the mitochondria, nucleus, junctional complex, lateral and basal cell membranes showed little or no stainability. Gastric mucosal homogenates were fractionated by centrifugation for further morphological and chemical analysis. The staining reaction of the microsomal fraction ($40,000 g \times 60 \text{ min}$) was similar to that of the tubular membranous components of intact oxyntic cells. Carbohydrate analyses showed that all cell fractions are extremely low in acidic sugars, uronic and sialic acids, while neutral sugars and hexosamines are relatively abundant. The microsomal fraction contains the largest proportion of carbohydrates, ca. 9% of the fat-free dry weight. Another distinguishing feature is that glucosamine is the only detectable hexosamine in the microsomal fraction. These histochemical and chemical data indicate that neutral glycoproteins are associated with membranous components which have been implicated in the process of HCl secretion by oxyntic cells. The staining pattern within the cells supports the hypothesis of interrelationships between the Golgi membranes, tubular smooth membranes, and apical surface membrane.

INTRODUCTION

The oxyntic or acid-secreting cells of the frog stomach are characterized by the possession of abundant smooth-surfaced tubular structures within the apical cytoplasm (1, 2). Parallel studies relating secretory activity to general morphological appearance have supported the proposal that these tubular membranous units are intimately concerned with the H^+ transport process of the stomach (2-5). Another point of evidence to favor this view was derived from our experiments with metamorphosing bullfrog tadpole stomachs in

which it was found that the developmental appearance of H^+ secretory capacity was temporally correlated with the morphogenesis of the tubular membranes within oxyntic cells (6). In this same study on tadpole stomachs, we presented evidence which suggested that the Golgi apparatus was in fact the cellular site for synthesis and elaboration of the tubular membranous elements which accumulated at the commencement of secretory activity.

On the basis of the available information, we

developed an hypothesis that the tubular membranes of mature oxyntic cells represent an intermediate morphological stage between their genesis in the Golgi region and their participation in gastric HCl secretion via intercommunication with the apical plasmalemma. One approach to ascertaining such interrelationships between various membranous components would employ specific cytochemical staining techniques on both intact tissue and cell membrane fractions.

The technique of Rambourg and Leblond (7) for glycoprotein staining was of particular interest since they found that outer cell membrane surfaces and inner aspects of the Golgi saccules were among the most reactive sites. These authors stressed the possible interrelationship of Golgi structures with the plasmalemma, especially with respect to synthesis of constituents of the cell coat.

In the present communication, we employ the periodic acid-silver methenamine staining reaction of Rambourg and Leblond (7) for investigating membranous structures of the bullfrog oxyntic cell. The histochemical procedures are coupled with morphological and chemical analysis of isolated cell fractions. We present evidence for a glycoprotein material which stains with equal intensity within the smooth-surfaced tubules, within Golgi saccules, and on the outer aspect of the apical cell membrane. The periodic acid-silver staining reaction of isolated gastric microsomes further supports the notion that this cell fraction is largely derived from the smooth tubules of oxyntic cells. Furthermore, carbohydrate analysis demonstrates the unique nature of the glycoprotein moieties associated with various gastric cell fractions.

METHODS

Fixation of Stomach Tissue

The frogs (*Rana pipiens* or *Rana catesbeiana*) were sacrificed by pithing, and the stomachs were immediately removed. The underlying muscle coat was stripped from the mucosa, and the latter portion was placed in a fixative of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.8, and minced into small pieces (less than 1 mm³). The pieces were placed in fresh glutaraldehyde fixative for an additional 60 min at room temperature. The tissue was then washed for 90 min in three changes of 0.1 M cacodylate buffer. Dehydration was carried out through a graded series of alcohol, and the tissue was finally embedded in Epon. Silver sections were cut with glass knives and mounted on uncoated, 200-mesh, stainless steel grids.

These grids were stored in a desiccator until subsequent staining procedures.

Staining Procedures: Periodic

Acid-Silver Methenamine

Sections were stained according to the method described by Rambourg (8). All staining was carried out at 60°C in a darkened room. The sodium borate, silver nitrate, and silver methenamine solutions were freshly prepared each time. Staining for 20 min with silver methenamine gave good positive results. Non-specific staining was assessed by deleting the periodic acid (P.A.) oxidation step in the treatment of some sections.

Periodic-Chromic Acid-Silver Methenamine

The method recently described by Rambourg, Hernandez, and Leblond (9) was followed, except that Epon and not Vestopol was used for embedding material. The sections were stained in silver methenamine for 40 min.

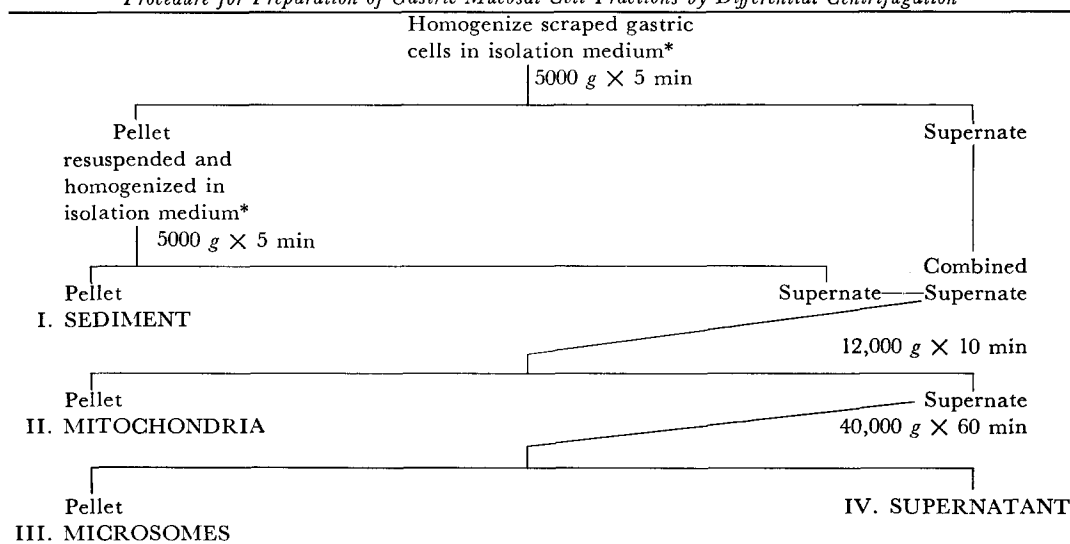
All sections were examined in the Hitachi HU 11 microscope.

Isolation of Cell Fractions from

Mucosal Homogenates

The gastric mucosa was obtained as described above, cut along the lesser curvature to form a sheet, and stretched out on filter paper. The mucosal, or secretory, surface was gently scraped, then blotted vigorously with filter paper so as to remove adhering mucus and some of the surface epithelial cells (10). The secretory surface of the fundic mucosa was then scraped vigorously with a stainless steel spatula. This removed most of the gastric glands from the underlying muscularis mucosae and connective tissue. The glandular scrapings from one or more stomachs were then triturated in a Potter-Elvehjem homogenizer with approximately 40 volumes of homogenizing medium. The latter was composed of 110 mM KCl, 10 mM Tris HCl, and 2 mM Na ethylenediaminetetraacetate (EDTA), all adjusted to pH 7.3. Fractionation by differential centrifugation is given in Table I. The four cell fractions were designated (I) sediment, (II) mitochondria, (III) microsomes, and (IV) supernatant. The cell fractions were subsequently used for ultrastructural and cytochemical studies or for chemical analysis. It is acknowledged that these cell fractions are not homogeneous with respect to cell organelles or their fragments. The method of separation is based primarily upon earlier work on enzymatic functions of gastric cell fractions (11-13).

TABLE I
Procedure for Preparation of Gastric Mucosal Cell Fractions by Differential Centrifugation



* Isolation medium contained 110 mM KCl, 10 mM Tris-HCl, 2 mM EDTA adjusted to pH 7.3.

Fixation

A small aliquot of an isolated cell fraction was placed in a conical BEEM¹ capsule, and an equal quantity of 2.5% glutaraldehyde in 0.1 M sodium cacodylate was added. The membranes were fixed in this fashion for 60 min and then centrifuged at 10,000 *g* for 10 min in order to pellet the material. The rest of the fixation was similar to that described for whole tissue, except that all steps were carried out in the conical BEEM capsules.

Negative Staining

A sample of the microsomal fraction, approximately 1 mg of protein/ml, was fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at 4°C. The fixed preparation was diluted with an equal volume of 2% sodium phosphotungstate, pH 7.4, and a small drop of this suspension was placed on a Formvar-carbon-coated grid. After 1 min, excess fluid was withdrawn with filter paper and the grid was immediately examined in the microscope.

Preparation of Cell Fractions for

Chemical Analysis

Cell fractions were treated with 0.3 M perchloric acid (PCA) and the resulting precipitates were washed

¹ Better Equipment for Electron Microscopy, Bronx, N.Y.

twice with PCA and twice with distilled water. The precipitates were dried (90°C overnight), weighed, treated with two washes of CHCl₃-CH₃OH (2:1), subsequently dried, and reweighed. The fat-free precipitates were digested with papain by suspension in a digestion medium containing 1 M NaCl, 0.1 M Na phosphate, 5 mM cysteine and 5 mM EDTA adjusted to pH 6.5. Approximately 85 mg of papain per mg fat-free dry weight was added to each tube according to the procedure of Pamer et al. (14). Digestion was carried out at 60°C for 24–48 hr with periodic adjustment of the pH of the medium back to pH 6.5 with NaOH. After digestion, PCA was added to a final concentration of 0.3 M and the tissue digests were centrifuged at 10,000 *g* for 10 min. The supernates were placed in washed dialysis tubing and dialyzed against 600 volumes of 0.02 M NaCl for 48 hr. The dialysates were collected and evaporated to dryness in vacuo over NaOH and P₂O₅. The dried residue containing carbohydrates and peptides (glycopeptide residue) was taken up in a volume of H₂O and stored at 4°C for further analysis.

Carbohydrate Analysis

Sialic acid was measured both on the “purified” glycopeptide residues and on the crude cell fractions prepared by differential centrifugation. The procedure of Warren (15) was used for these assays. Neutral sugars were analyzed according to the methods described by Dische (16). D-Glucose and L-fucose were used as the respective standards. The neutral sugar

analyses were carried out both directly on the glycopeptide residues and on aliquots hydrolyzed in sealed vials containing 1 M HCl at 100°C for 4 hr. The latter procedure resulted in a destruction of some 25–50% of hexose, and thus was discontinued.

Uronic acid was determined by the method of Bitter and Muir (17), using D-glucuronolactone (CalBiochem, Los Angeles, Calif.) and chondroitin sulfate "C" (CalBiochem) as standards.

For hexosamine determinations, aliquots of the glycopeptide residues were sealed under vacuum in vials containing 6 M HCl. Hydrolysis proceeded at 95°C for 4 hr, and the resulting hydrolysate was evaporated to dryness in vacuo over NaOH and H₂SO₄ at room temperature. The hydrolysates were again evaporated to dryness after approximately 2 ml of H₂O was added to each sample. Hexosamine was determined either directly on the hydrolysate by Elson-Morgan method (18) or after the chromatographic purification described by Boas (19). The effectivity of hydrolysis and the ultimate yield of the assay procedures were ascertained by standards of chondroitin sulfate and internal standard of D-galactosamine-1-¹⁴C which were carried on through the entire hydrolysis and purification procedure. These tests showed at least 90% recovery for hexosamines. The ratio of glucosamine:galactosamine was determined by the borate depression method of Tracey (20).

RESULTS

Morphology

The fine structure of oxyntic cells fixed only with glutaraldehyde is seen in Fig. 1. Although over-all preservation is excellent, there appears to be some vacuolization especially within the apical region of the cells. The lumen of the gastric gland is partially occluded by cytoplasmic extensions from the apical surface of the cells: these surface extensions have often been described as microvilli (2, 3, 5). An outstanding feature within the oxyntic cells is the abundance of tubular smooth membrane profiles seen both in cross-section and in longitudinal section. The tubular elements are packed extremely closely and in a very regular pattern as is evident from the hexagonal array indicated by the arrows in the micrograph. Mitochondria are well preserved but appear in negative contrast since the cristae have little electron opacity without osmium tetroxide postfixation. The rough endoplasmic reticulum is interspersed with the mitochondria in the more basal regions of the cells. Selected areas of the oxyntic cell are

shown at higher magnification in Fig. 2 *a-c*. It is apparent that the staining intensity of specific cell structures differs considerably with glutaraldehyde as the sole fixative, as compared to previous work in which aldehyde fixation was followed by osmium tetroxide postfixation (2, 5, 21).

A section through a region dense with tubules is shown in Fig. 2 *a*. Inspection of transverse profiles of tubules reveals that the membrane is a trilaminar structure: an electron-opaque inner leaflet adjacent to the tubular lumen; an electron-transparent region; and a dense outer leaflet. The cytoplasmic regions between the tubules appear quite dense under these conditions of fixation, as compared to those which were postfixated in osmium tetroxide, and the outer leaflet of the tubule often blends with the cytoplasmic density. The mean over-all diameter of the tubule is about 360 Å, whereas the diameter across the inner dense leaflet of the tubule is 200 Å. The center-to-center spacing from one tubule to another in hexagonally packed regions is approximately 400 Å.

A region from the more basal portion of an oxyntic cell is shown in Fig. 2 *b*. The cisternae of the rough endoplasmic reticulum appear to have a dense core which may result from the apposition of the inner faces of the cisternal membranes. Dense, round ribosomal particles can be seen lining the outer surfaces of the membranes.

Junctional complexes between adjacent oxyntic cells often show interesting detail after only glutaraldehyde fixation, as seen in Fig. 2 *c*. A number of electron-opaque filamentous structures may be seen joining the adjacent faces of the desmosome. These structures, which are ordinarily not seen after osmium tetroxide postfixation, have rather regularly spaced intervals of approximately 100 Å.

P.A.-Silver Methenamine Staining

Sections oxidized with periodic acid were intensely stained after silver reduction.

This phenomenon is seen in Fig. 3 which shows parts of a lumen surrounded by several oxyntic cells. It is clearly evident that the luminal surface membranes and apical cytoplasmic membranes of these cells are heavily stained. By nature of the staining procedure, i.e. no osmium or counterstaining with uranyl and lead salts, it is not possible to visualize the individual profiles of the cytoplasmic tubules; however, the strandlike deposition of reduced silver indicates that this is

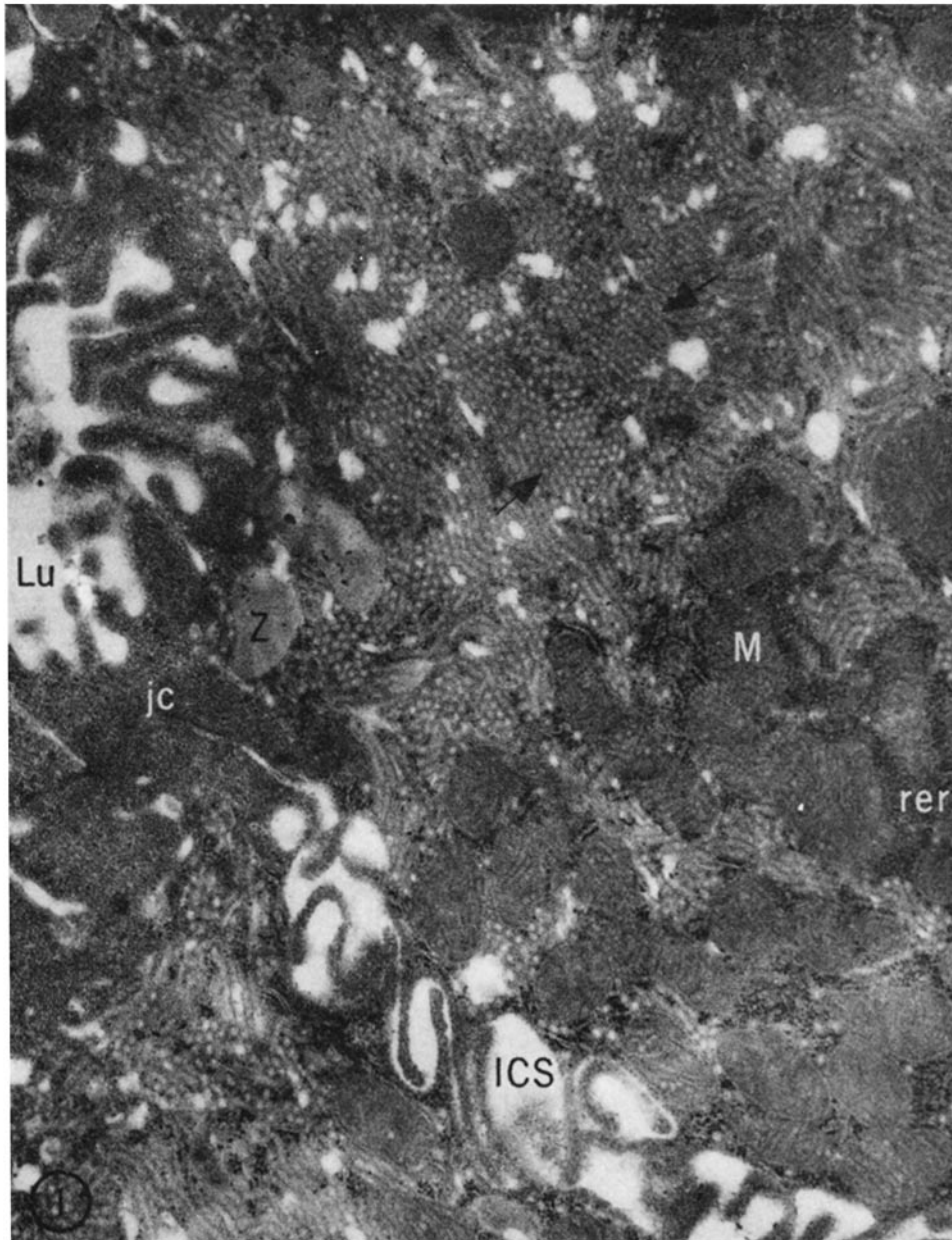


FIGURE 1 Section through a portion of two oxyntic cells which have been fixed with only glutaraldehyde. Numerous irregular cytoplasmic extensions project into the gland lumen (*Lu*). Just beneath the apical surface is a cytoplasmic region relatively free of structures, and adjacent to this region are innumerable profiles of tubular smooth membranes seen both in transverse and in longitudinal aspects. The hexagonal packing of the tubular structures is indicated by arrows. Mitochondria (*M*) and the rough-surfaced endoplasmic reticulum (*rer*) are generally confined to the more basal portion of the cell. *ICS* refers to the intercellular space while *jc* indicates the junctional complex. An occasional zymogen granule (*Z*) can also be seen. Section was stained with uranyl and lead salts. $\times 33,600$.

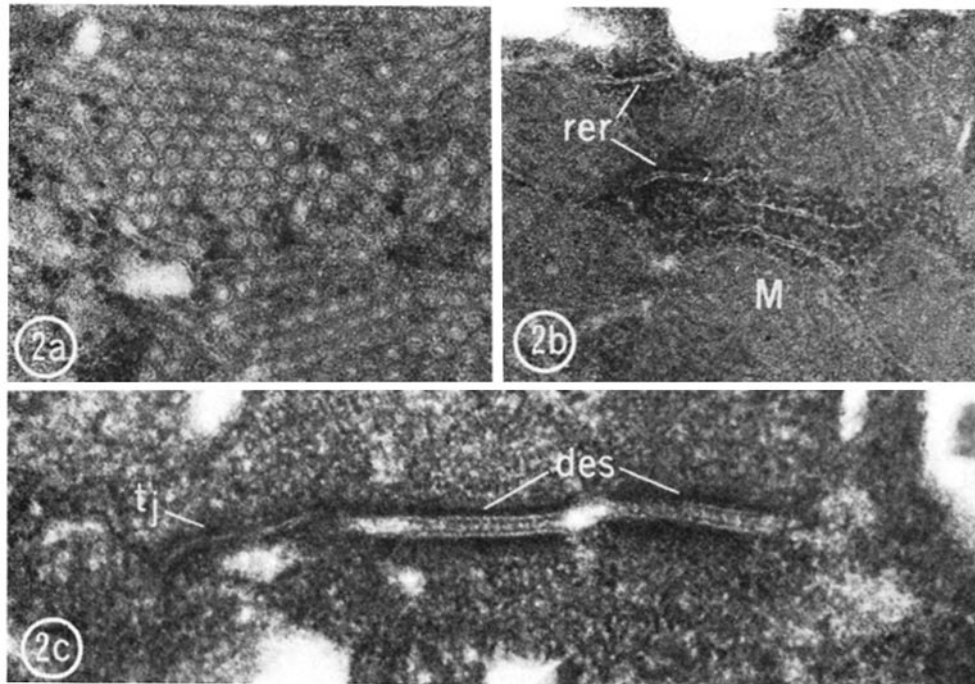


FIGURE 2 An enlarged view of selected areas from oxyntic cells fixed with only glutaraldehyde. Fig. 2 *a*, Section through smooth tubular elements in the apical region of the cell. The tubule membranes are trilaminar structures consisting of two dense layers separated by an electron-transparent middle layer. Lumina of the tubules are electron transparent while the surrounding cytoplasm is relatively dense. The hexagonal arrangement of the tightly packed tubules is also seen with a center-to-center distance of approximately 400 Å. $\times 79,500$. Fig. 2 *b*, More basal region of oxyntic cell showing rough-surfaced endoplasmic reticulum (*rer*) and mitochondria (*M*). The outer surface of the cisternae of the *rer* are studded with dense ribosomal particles, and a dense line, probably representing apposed membranes, can be seen within the cisternae. The mitochondria, whose cristae are far less dense than the surrounding protein matrix, appear in negative contrast compared to conditions in which postosmication is employed. $\times 56,000$. Fig. 2 *c*, A section through a junctional complex between two oxyntic cells. The tight junction (*tj*) displays the usual pentalaminar structure. Adjacent faces of the desmosome (*des*) are joined by fine, dense, filamentous structures which are spaced at rather regular intervals. $\times 111,900$. All sections were strained with uranyl and lead salts.

indeed the tubular membrane system. The mitochondria, on the other hand, are almost devoid of stain. The tight junctions, lateral borders, and the basal membranes including the basal infoldings are also only lightly stained. The basement membrane of oxyntic glands shows little affinity for the stain; this is in contrast to the heavily reactive basement membrane shown by Rambourg and Leblond for several epithelial tissues (7). Some of the stained structures, however, such as the nucleus, ribosomes, and extracellular collagen, were nonspecifically stained as judged by silver methenamine treatment of nonoxidized sections.

The apical portion of an oxyntic cell is seen in

greater detail in Fig. 4. The region just beneath the surface is almost devoid of structures; this corresponds to the cytoplasmic zone frequently seen in conventionally stained sections (cf. Fig. 1). Occasional heavily stained vesicles are seen within the clear zone, and these profiles probably represent oblique sections through surface infoldings. The bulk of the cell is intensely stained in the apical region corresponding to the location of the smooth-surfaced tubular membranes. The reduced silver grains appear spherical or as beaded strands suggesting transverse or longitudinal sections through the tubular system.

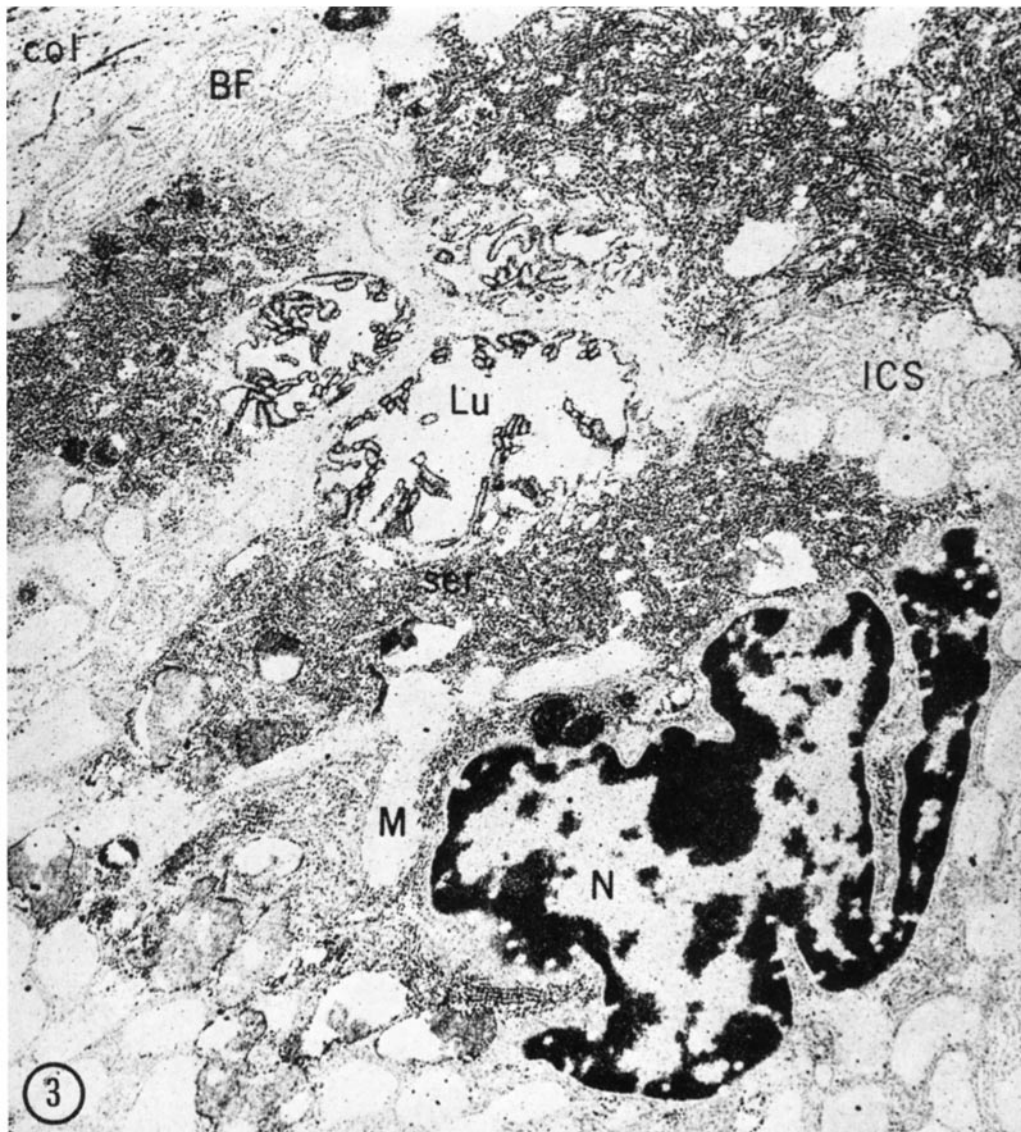


FIGURE 3 Transverse section through a portion of a gastric gland treated with periodic acid-silver methenamine. Apical cytoplasmic extensions, whose surfaces are intensely stained, can be seen lining the gland lumen (*Lu*). The apical portion of the cell corresponding to the tubular smooth-surfaced membranes (*ser*) is also heavily stained. Mitochondria (*M*) are almost devoid of stain while the nucleus (*N*) and extracellular collagen (*col*) are nonspecifically stained. The lateral cell borders (*ICS* region) and basal membrane infoldings (*BF*) are only moderately stained. $\times 15,000$.

The qualitative difference in stainability of the various regions of the surface membranes is strikingly evident in Fig. 4. The apical portions of the plasma membrane avidly reduce silver methenamine; on the other hand, the lateral and basal

surfaces are much less intensely stained while the tight junction is almost devoid of stain. Such variations in staining of the cell surface suggest differences in the chemical nature of various portions of the cell membrane.

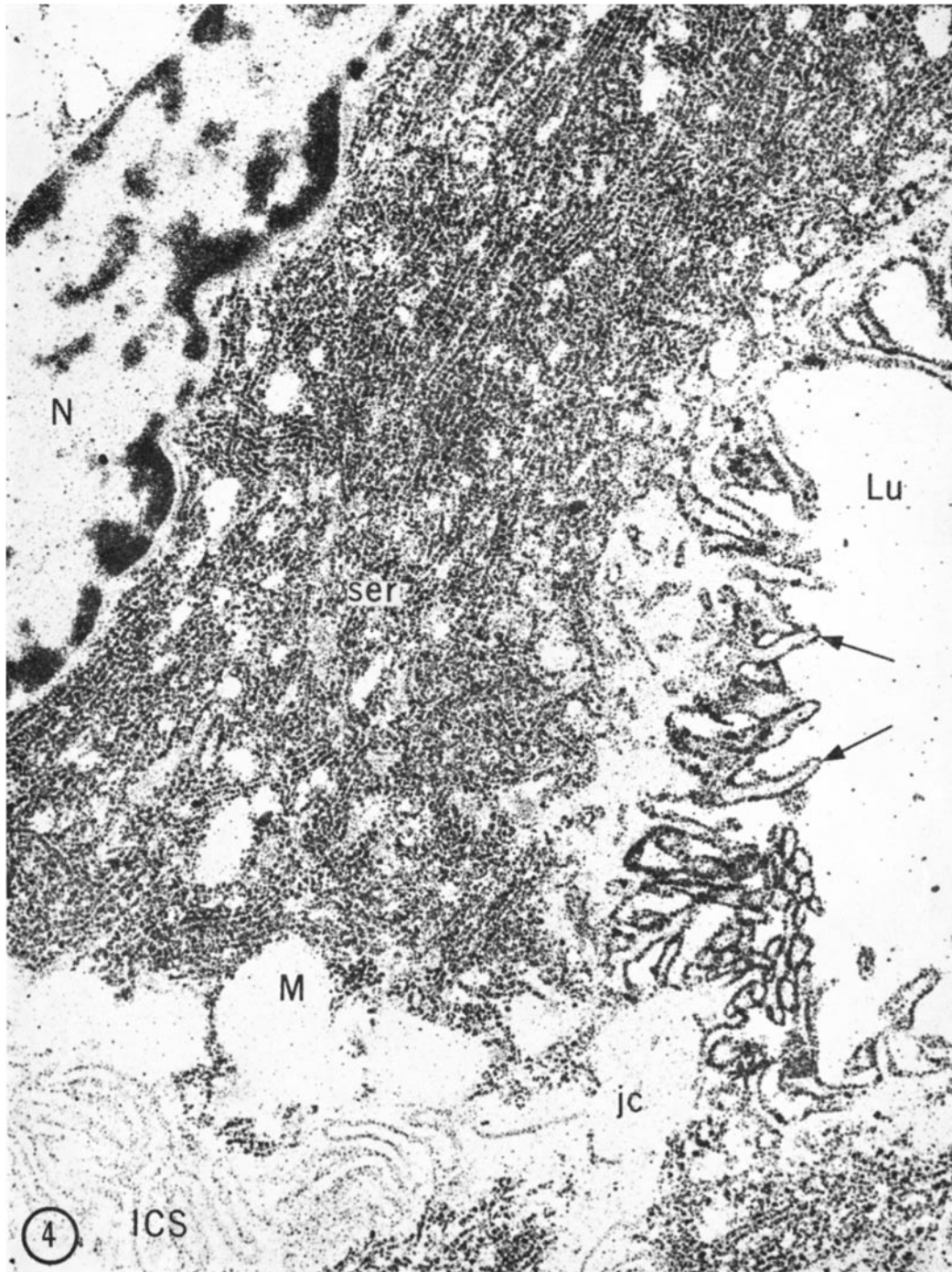


FIGURE 4 Periodic acid-silver methenamine-stained section of the anterior portion of an oxyntic cell. The lumen (*Lu*) is richly lined with cytoplasmic extensions (arrows) whose surfaces display the same intense staining reaction as the region corresponding to the tubular smooth-surfaced membranes (*ser*). The area just beneath the luminal surface is relatively free of stain except for occasional, strongly reactive, irregularly shaped vesicular structures which are probably oblique sections through the irregular surface. The mitochondria (*M*) and junctional complex (*jc*) are almost devoid of silver grains, whereas the lateral cell membranes (*ICS*) are moderately stained. $\times 28,500$.

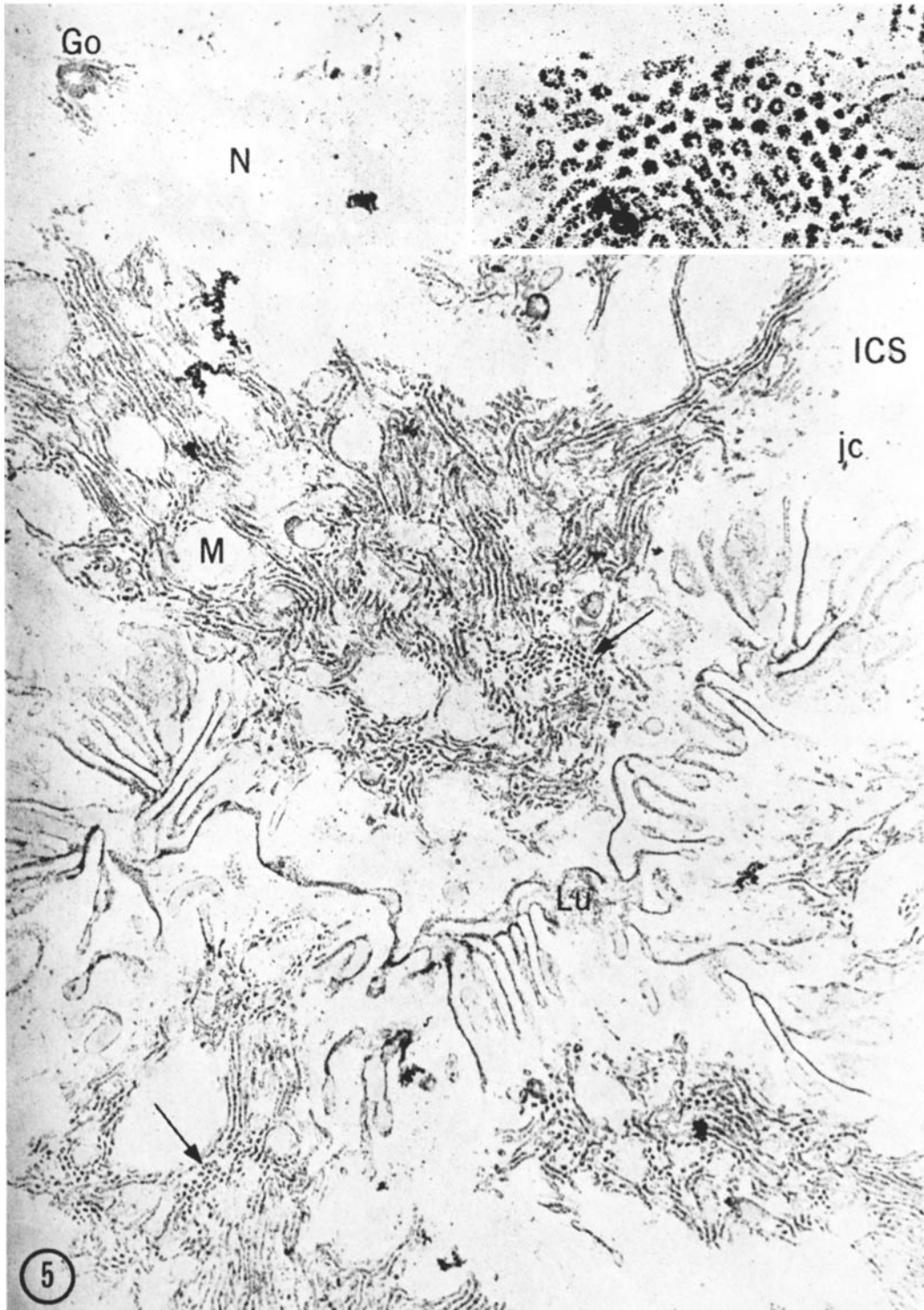


FIGURE 5 Periodic acid-chromic acid-silver methenamine-stained section through several oxyntic cells. The lumen (*Lu*) is almost completely occluded by the apical cytoplasmic extensions which are heavily stained. Vesicular structures just beneath the surface are also stained. The internal aspects of the tubular smooth membranes are intensely stained in both their longitudinal and transverse profiles. Regions showing hexagonal packing are indicated by arrows. The Golgi apparatus (*Go*) is highly reactive whereas nuclei (*N*), mitochondria (*M*), lateral cell membranes (*ICS*) and junctional complexes (*jc*) show little or no reactivity. $\times 28,500$. The *inset* shows in greater detail the structure and arrangement of the hexagonally packed tubular smooth membranes where a center-to-center spacing of 400 Å is found (cf. Fig. 2 *a*). $\times 79,500$.

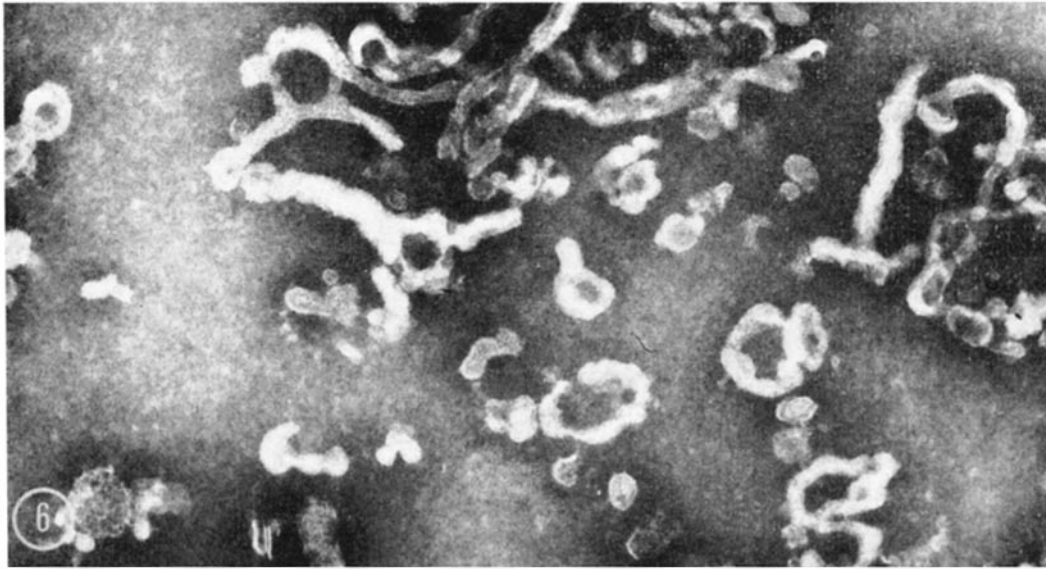


FIGURE 6 Tubular and vesicular structures from the microsomal fraction of the frog stomach seen in negative contrast after sodium phosphotungstate staining. $\times 58,500$.

*P.A.-Chromic Acid-Silver
Methenamine Staining*

Rambourg, Hernandez, and Leblond (9) demonstrated that when chromic acid oxidation was used in conjunction with periodic acid the staining reaction with silver methenamine was far more specific and sensitive. This was borne out in our preparation as typified in Fig. 5. Nuclear and ribosomal stainability are very much reduced. For the oxyntic cells shown in Fig. 5, the glandular lumen is almost completely occluded by the apical projections which are intensely stained at their surfaces. Just beneath the surface, there is a relatively clear area with a few vesicular structures which are also stained and probably represent oblique sections through the folds of the highly irregular surface. Densely stained tubular elements of the smooth-surfaced membranes in both longitudinal and transverse aspects are seen throughout the apical portions of the cell; in fact, in some areas the hexagonal packing of the tubules is clearly evident with a center-to-center spacing of approximately 400 A. In cross-section, the tubules appear to contain a ring of dense silver granules; in some instances, the tubular lumina are also filled with stain (Fig. 5, *inset*). Although a fine structural analysis of stain distribution is difficult

owing to the large grain size of reduced silver particles, we have measured the outer diameter of the dense outlines in areas of hexagonal packing. The mean diameter was 280 A. Since the outer reaches of the distribution of stain approximately correspond to the position and dimensions of the middle, electron-transparent lamina of the tubule membrane, it is likely that the reactive chemical groups are predominantly associated with the inner aspects of the tubular membrane.

The Golgi apparatus which is found close to the nucleus is also highly reactive with silver methenamine. Stain is found associated with both the cisternal and vesicular elements of the Golgi saccules. Mitochondria membranes and lateral and basal plasma membranes, on the other hand, have little affinity for the stain. As was the case in Figs. 3 and 4, the junctional complex is barely discernible.

Staining Properties of Isolated Cell Fractions

The microsomal fraction visualized after negative staining (Fig. 6) contains tubular structures which often form closed loops. Occasionally, however, vesicular structures can also be seen. Such variously shaped elements are also found in sections of the microsomal pellet which has been

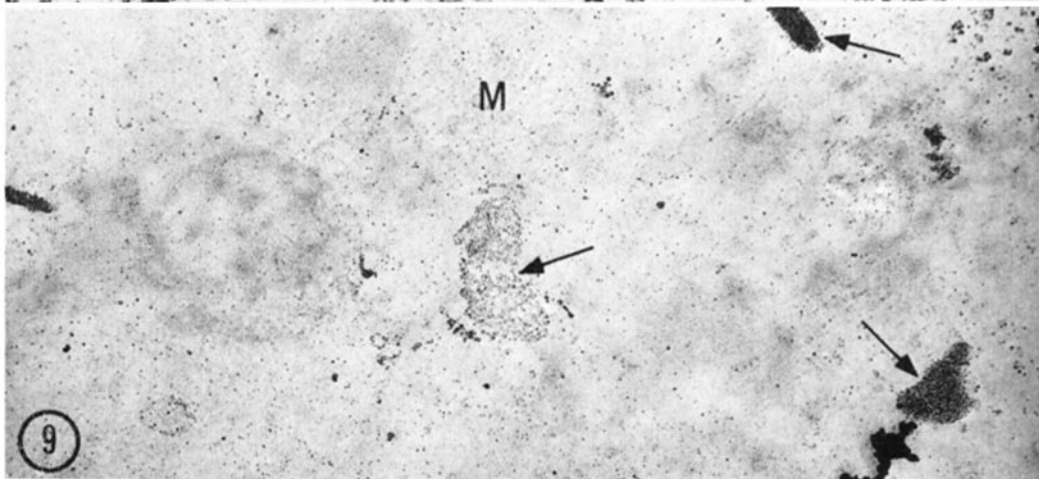
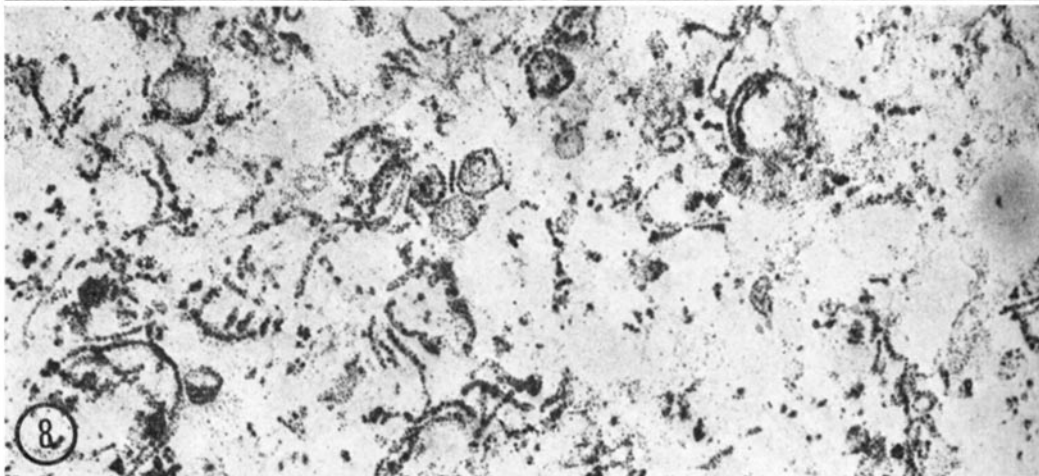
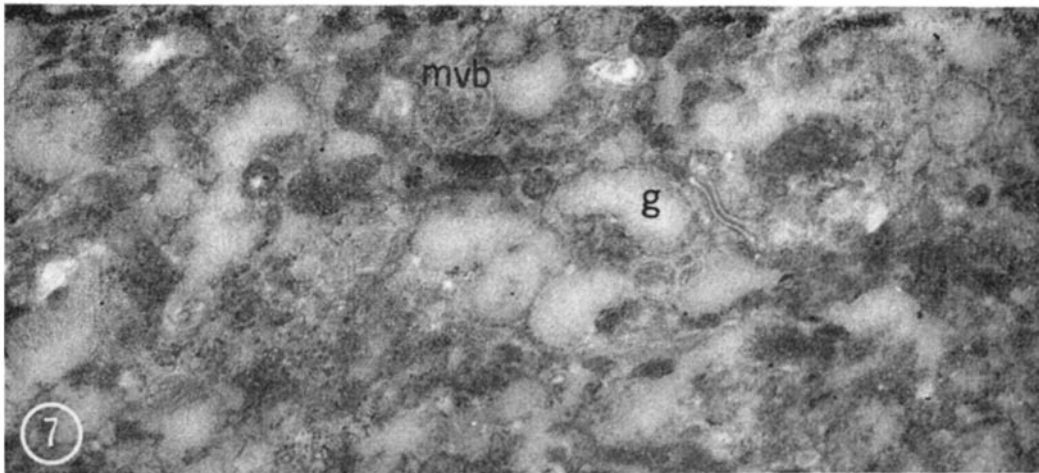


FIGURE 7 Section through a glutaraldehyde-fixed microsomal pellet. Numerous lamellar and vesicular structures are present; however, other structures such as multivesicular bodies (*mvb*) and clear, ovoid granules (*g*) can also be seen. Section was stained with uranyl and lead salts. $\times 44,400$.

FIGURE 8 Same microsomal preparation as in Fig. 7, except that the section was stained with periodic acid-chromic acid-silver methenamine. There are abundant highly reactive strands and rounded masses which correspond to the membranous lamellae and vesicles seen in the previous micrograph. The large ovoid transparent areas probably identify the region of the granules seen in Fig. 7. $\times 44,400$.

FIGURE 9 Periodic acid-chromic-silver methenamine-treated section of glutaraldehyde-fixed mitochondrial fraction. Outlines of mitochondria (*M*) are barely discernible by this staining technique. The dense granules (arrows) may represent fragments of adhering mucus. $\times 42,000$.

TABLE II
Carbohydrate and Lipid Analyses on Cell Fractions Derived from Bullfrog Gastric Epithelium

Fraction*	I Sediment	II Mitochondria	III Microsomes	IV Supernatant
Fat-free dry wt (mg)	231.7	14.4	22.4	535.1
Total lipid wt (mg)	63.2	16.7	29.3	8.9
Phospholipid‡ (mg)	20.9	2.6	12.3	2.6
Cholesterol (mg)	15.3	1.3	10.3	2.6
Carbohydrate analyses ($\mu\text{g}/\text{mg}$ fat-free wt)				
Total hexosamine	40.1	38.4	33.0	2.9
Glu-NH ₂ §	23	82	100	78
Gal-NH ₂	77	18	0	22
Hexose	17.9	19.1	39.1	2.2
Fucose	8.3	8.7	17.3	0.4
Sialic acid	0.4	0.4	0.7	0.01
Uronic acid	0.3	1.4	1.6	0.1

*In experiment reported here, fractions were derived from a total wet cell mass of 3.15 g.

‡Calculated from inorganic P analysis, assuming an average phospholipid mol wt = 750.

§Ratio of glucosamine/galactosamine estimated by the borate depression method of Tracey (20).

fixed with glutaraldehyde and positively stained with uranyl and lead salts as shown in Fig. 7. In addition, other structural components such as clear granules and multivesicular bodies are also found in the pellet.

When thin sections of the microsomal pellet are treated with P.A.-chromic acid-silver methenamine, many of the structural elements become intensely stained (Fig. 8). Strandlike and vesicular depositions of silver predominate in the field. In their size and shape, these stained structures are comparable to the densely stained tubular and vesicular elements of oxyntic cells noted above (cf. Fig. 5). Certain nonreactive areas are vaguely outlined and probably correspond to the granules seen in Fig. 7. The lack of stain indicates that these latter structures are not mucous granules. By comparison, the staining pattern is quite different for the mitochondrial fraction shown in Fig. 9. There is very little stainable material in the mitochondria whose outlines appear extremely vague. There are a few heavily stained granules apparent in the mitochondrial fraction, and these probably represent contaminating mucous granules or their fragments.

Carbohydrate Analysis of Isolated Mucosal Cell Fractions

The results of chemical analyses on various cell-fractions derived from gastric mucosal homogenates are shown in Table II. Although the carbohydrate analyses are the primary concern here, measurements of lipid content are included for a more complete evaluation of the cell fractions. Clearly, the mitochondrial and microsomal fractions contain the highest proportion of lipids, consistent with their membranous nature.

Carbohydrates represent a sizeable proportion of the fat-free mass of the sediment, mitochondrial, and microsomal fractions. The microsomes are the most extreme case, in that the sugars (Table II) represent about 9% of the fat-free dry weight. It should be pointed out that much of the polysaccharide of frog gastric homogenates is derived from mucus from residual surface epithelial cells and mucous neck cells. We have not ascertained the extent to which various cell fractions are composed of these secretory mucins.

In general, the analytical data indicate that there is abundant glycoprotein material in frog

gastric tissue. The acidic sugars, uronic, and sialic acids, are found in rather low concentrations in all cell fractions. In contrast, the neutral sugars, hexoses, and methylpentoses, are relatively abundant in all fractions and especially prevalent in the microsomal fraction. Amino sugars also represent a major carbohydrate component. Characteristic distinctions occur in the ratio of glucosamine: galactosamine for each fraction. It is of interest that no detectable galactosamine was observed in the microsomes.

DISCUSSION

Nature of the P.A.-Silver Methenamine-Staining Reaction

Through the use of various staining techniques, a cell coat which is rich in carbohydrate has been demonstrated for many vertebrate cells (7). The cytochemical silver-staining reaction used in this work has been attributed to the reduction of the silver tetramine by the aldehydic groups produced via oxidation of 1,2-glycols and α -amino alcohols by periodic and chromic acids (9). Glegg, Clermont, and Leblond (22) had shown earlier that neutral sugars are readily stainable whereas acidic mucopolysaccharides, such as heparin and chondroitin sulfate, are not reactive. In their application of the silver methenamine-staining technique to numerous tissues, Rambourg, Hernandez, and Leblond (9) demonstrated that the reaction was most pronounced at the cell surface, the inner aspects of the Golgi apparatus, and in vesicular structures having their origin from the Golgi region. From the results of "in vitro" studies on the stainability of various carbohydrates, these authors felt that glycoproteins were most likely to contain the reactive groups which take up stain in the cell structures.

Histochemical tests on gastric mucosa with the light microscope (23, 24) and at the ultrastructural level (25) demonstrate that oxyntic cells contain essentially no acid mucosubstance. Although acidic glycoproteins, such as sialoproteins and sulfated glycoproteins, have been localized in the gastric surface epithelial cells, the only fundic cell type in which a characteristic mucopolysaccharide occurs is the mammalian chief cell (23, 24). The low levels of uronic acids observed in the frog fundic mucosal cell fractions are consistent with the histochemical data,

especially since a typical chief cell is not present in the main body of amphibian stomachs (26).

On the other hand, periodic acid-Schiff staining of acid-secreting cells from mammalian stomach has been demonstrated by several groups (23, 24). In these reports, there were no accompanying fine structural studies, and thus it was not possible to localize the intracellular site of the stain. Recently, Sedar (25) has shown that a polysaccharide substance, probably a neutral carbohydrate, lines the smooth-surfaced membrane system and outer cell coats of parietal cells of the rat after phosphotungstic acid staining. The staining pattern which we have demonstrated with P.A.-silver methenamine in oxyntic cells of the frog is similar to that described by Sedar. The bulk of evidence, both histochemical and direct analytical, indicates that the reactive material is glycoprotein.

Interrelations between Membrane Structures

The fact that both the cytoplasmic tubules and apical surface membranes have similar staining properties with the P.A.-silver methenamine reaction makes the general hypothesis of membrane transformations between these two components more plausible. We attempted to evaluate the specific location of the stain on the apical plasmalemma. In untreated sections, similar to that shown in Fig. 1, the combined thickness of the interspace plus the adjacent cell membranes on either side of closely apposed cytoplasmic extensions was found to be 200–250 A. In sections stained by the P.A.-chromic acid-silver methenamine procedure, we found that a single dense line of silver grains, 70–100 A thick, is seen at the interface between closely apposed apical cell membranes (cf. Fig. 5). This single line of stain is too narrow to encompass both cell membranes and the interspace, and thus it is likely that the reacting carbohydrate groups are on the outer aspects of the apical plasmalemma. This and similar arguments have been used by Rambourg and Leblond (7) to localize carbohydrate materials of the cell coat. Specific deposition of the stain on the outer faces of the apical plasmalemma and at the inner aspect of the tubular smooth membranes is consistent with several schematic interpretations of the morphological events which accompany stimulation of HCl secretion (2, 27).

The positive reaction of Golgi membranes with silver methenamine is consistent with widespread reactivity of Golgi structures reported by Ram-

bourg, Hernandez, and Leblond (9). In a previous study on the development of tadpole gastric mucosa, we presented morphological evidence to suggest that the Golgi apparatus was the source of tubular membranes found in mature oxyntic cells (6). The similar silver-staining reactivity of the respective membranous components supports this proposed origin. However, such evidence is not intended to imply identical membrane composition but merely similarity of specific chemical groupings.

Carbohydrate Staining in Isolated Cell Fractions

The microsomal fraction of gastric mucosal homogenates gives a positive reaction with the P.A.-silver methenamine staining procedure and the pattern of silver deposition is similar to that of the tubules seen in intact oxyntic cells. Thus, it is reasonable to speculate that the microsomal fraction is largely derived from these cellular structures. This supports previous evidence based upon morphological comparison (12), and permits an interpretation of the chemical and enzymatic composition of gastric microsomes in terms of intact cell components. However, contamination of the microsomes with other cell constituents is a recognized problem in the evaluation of chemical data.

Nature of the Carbohydrate Components

Although a great deal is known about the carbohydrate composition of mucins secreted by the stomach, relatively little information is available on the sugar content of gastric tissue or cell fractions derived therefrom. Pamer, Glass, and Horowitz (14) have given a detailed analysis of the acidic polysaccharides of rabbit gastric mucosa. However, their results are not comparable to those reported here because their procedures were not intended to recover neutral polysaccharides or glycoproteins. In a recent report, Nemoto and Yosizawa (28) succeeded in isolating four oligosaccharide fractions from rabbit gastric mucosal digests: these consisted of a glycopeptide moiety and three acidic glycosamino-glycan (mucopolysaccharide)-peptides. Our studies are in general agreement with the analytical data of Nemoto and Yosizawa, although a detailed comparison is not possible since their analytical sugar data are reported as a fractional per cent of each polysaccharide subfraction. There is a considerable quantity

of glycoprotein in frog gastric mucosa. On the other hand, the levels of acidic glycosaminoglycans in various cell fractions are rather low (less than 10% of total gastric tissue carbohydrates). Again, it may be pointed out that such differences in distribution of neutral and acidic carbohydrate units between amphibian and mammalian (rabbit) gastric mucosa are consistent with histochemical evidence cited earlier that abundant acidic mucopolysaccharide can be demonstrated in the mammalian chief cell only.

Of the various cell fractions isolated from gastric mucosal homogenates, the microsomes are of special interest since (a) they appear to be derived from the tubular membrane system of oxyntic cells; (b) morphological evidence implicates a role for this tubular membranous system in the HCl secretory process (2-5); and (c) enzyme systems, indirectly linked to H⁺ secretion, have been localized in the microsomal fraction (11-13). Thus, an insight into the chemical nature and macromolecular architecture of the microsomal membranes may prove useful for interpreting the mode of their participation in gastric secretory activity. The glycoprotein layer which has been histochemically localized in the tubular membranous system is one such structural and chemical entity.

The level of the present results does not permit an assessment of the number and composition of specific glycopeptides within the gastric cell fractions. However, the homogeneity of hexosamine (glucosamine) in the microsomes suggests that a single major glycoprotein might be present in this fraction. The high content of fucose, especially in the microsomal fraction, warrants the general classification of frog gastric glycoprotein into the fucoprotein category. On the other hand, sialic acid represents a relatively low proportion of gastric membrane carbohydrates, a situation which is quite different from that of several other membrane systems (29-31).

The function of the stainable glycoprotein layer on the oxyntic cell membranes is as yet unknown. Several possible modes of involvement with the secretory activity of oxyntic cells may be proposed, among which are (a) the glycoprotein may facilitate membrane transformations during active HCl secretion, perhaps by serving as a "recognition" factor at specific fusion points, (b) the carbohydrate moieties may operate as protective groups to prevent denaturation of membrane

proteins during H⁺ production, (c) the carbohydrates may have a direct role in the translocation of H⁺. It had been proposed earlier that a mucosubstance within the tubular membranous system might act as an anionic exchange matrix for H⁺ secretion (2, 32). The paucity of negatively charged carbohydrate moieties within the gastric membrane glycoprotein precludes such a possibility.

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