

ELECTRON MICROSCOPE OBSERVATIONS ON THE CARBOHYDRATE-RICH CELL COAT PRESENT AT THE SURFACE OF CELLS IN THE RAT

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ABSTRACT

Periodic acid-silver methenamine, a fairly specific technique for glycoprotein detection, was used to stain a variety of rat tissues, in the hope of confirming the existence of a carbohydrate-rich "cell coat" at the surface of mammalian cells. It was found that nearly all cells are coated by a thin layer of stained material. Around fibrocytes and migrating blood cells, the layer is uniform and merges with the ground substance. In the nervous system, cells and processes are surrounded with a layer whose density increases in synaptic clefts. Around epithelial cells, the layer outlines apical microvilli, follows lateral interspaces, and extends between cells and basement membrane. The layer is continuous with the middle plate of desmosomes and can be followed within the wide portion of terminal bars. In contrast, staining usually vanishes when two adjacent plasma membranes fuse to form tight junctions. These findings indicate that the stained layer is a "cell coat" located outside the plasma membrane. Since the cell coat is also stained by colloidal thorium, a technique for detection of acidic carbohydrates, this structure presumably contains not only glycoprotein(s) but also acidic residues. The carbohydrates may play a role in holding cells together and in controlling the interactions between cells and environment.

Many years ago Chambers described one or more layers of organic material coating the plasma membrane of some invertebrate eggs (19) and protozoa (20). Such "extraneous coats" were not observed around vertebrate cells, but Chambers proposed that these cells are embedded in a jelly-like cementing substance (20).

Recently, the presence of carbohydrate was detected in the extraneous coats of invertebrate eggs (81) and protozoa (3, 77, 88, 92, 111). As for vertebrate cells, the situation was not clear and the information available was largely of an indirect nature. For example, when embryos or tissue cultures were treated with trypsin, the resulting dissociation of the cells seemed to be due to removal of a "proteinaceous" or "mucinous" material embedding them (30, 83, 100, 123). In fact, the electron microscope showed at the surface

of some cells a thin layer of amorphous material which, according to Bennett (7), corresponds to the carbohydrate-rich coat of protozoa and might exist on all cell surfaces, although "at the present, available evidence does not permit such a conclusion." In 1962, Gasic provided the first direct histochemical evidence of a carbohydrate-rich layer at the surface of some mammalian cells (40, 41, 101); but, perhaps because his work was chiefly done with ascites tumor cells, the opinion arose that surface carbohydrates are a feature of malignant cells (106). This opinion persisted in spite of a few dissenting voices, such as that of Kalckar who thought that carbohydrates may be "as abundant, if not more so, in normal cells" as in tumor cells (54). The situation was clarified when we recently observed in the rat that some 50 different cell types are coated with a thin layer of material

stained by the periodic acid-Schiff technique (which mainly detects glycoproteins) and the colloidal iron-Prussian blue technique (which detects acidic groups, primarily those in carbohydrates); and we concluded *not only that the presence of a "cell coat" is a common feature of vertebrate cells, but also that this coat is rich in carbohydrates* (98).

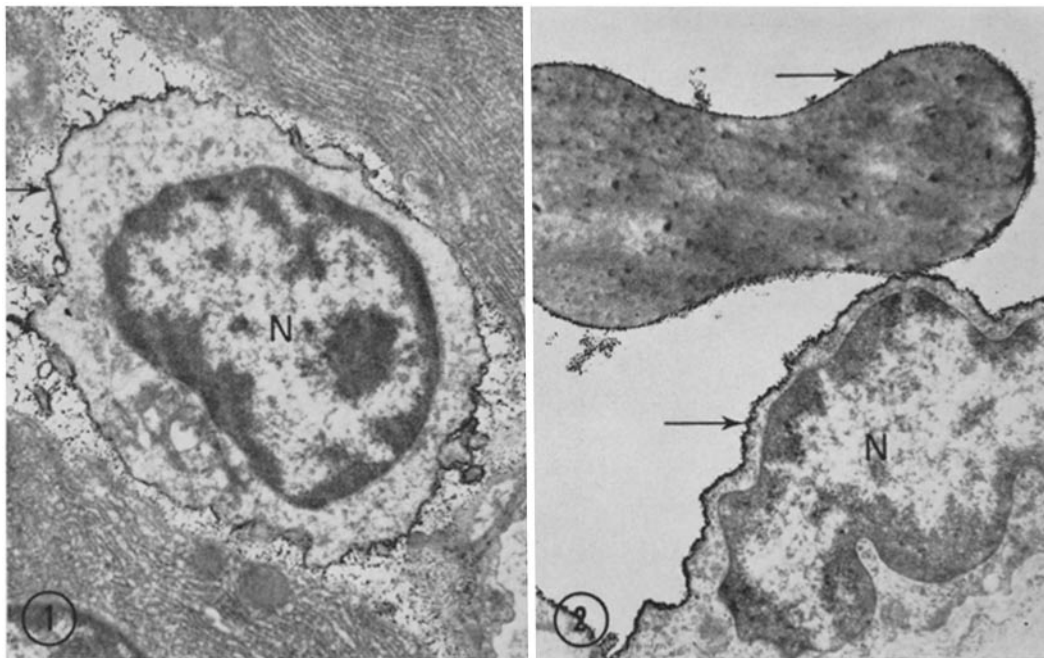
These results were obtained with the light microscope. In the hope of confirming them with the electron microscope, we used colloidal thorium instead of colloidal iron (99); and the periodic acid (PA)-Schiff sequence was replaced by PA-silver methenamine, a technique which yields a silver precipitate visible in the electron microscope (22, 28, 47, 53, 76, 78, 84, 91, 110, 116). Fixation in glutaraldehyde instead of osmium tetroxide or formaldehyde improved the quality as well as specificity of this technique. The colloidal thorium

and especially the PA-silver technique made it possible to confirm the existence of a "cell coat" covering mammalian cells and to further our understanding of this structure.

METHODS

Colloidal Thorium

Small pieces of organs and tissues from adult rats were fixed for 2 hr in a 2.5% glutaraldehyde solution buffered at pH 7.2 (48, 122), washed in phosphate buffer at pH 6.9 and postfixed for 2 hr in 2% osmium tetroxide at pH 7.2, all these steps being carried out at 4°C. After several rinses in phosphate buffer and one rinse in 3% acetic acid (pH 2.6), the specimens were immersed for 24 hr at room temperature in a 1% colloidal thorium solution in 3% acetic acid (pH 2.6). They were then rinsed again in 3% acetic acid. Following dehydration and Epon embedding, silver-



Electron microscope views of various tissues stained with colloidal thorium.

FIGURE 1 *Lymphocyte.* Thorium particles are accumulated at the surface of the lymphocyte whose nucleus (*N*) is seen in center. As a result, a heavy dark line (arrow) follows the plasma membrane. Colloidal particles are also present in the connective tissue space which surrounds this cell. This space separates two pancreatic acinar cells, the ergastoplasm of which may be identified in the upper right and lower left corners. $\times 12,000$.

FIGURE 2 *Red blood cell.* A layer of colloidal particles (arrows) may be seen at the surface of the red blood cell in the upper part of the picture and at the apical surface of the endothelial cell whose nucleus is visible at lower right (*N*). $\times 18,000$.

to-gold sections were cut, placed on uncoated grids, and counterstained with uranyl acetate.

Periodic Acid-Silver Methenamine

Small pieces of adult rat tissues were fixed in 2.5% glutaraldehyde as described above. In addition, incisor teeth fixed by perfusion with this glutaraldehyde solution were decalcified (122) and kindly placed at our disposal by Dr. Warshawsky. After Epon embedding, silver-to-gold sections were prepared, and stored by floating on 10% aqueous acetone. For staining, the sections were scooped out of this solution with a clean wire loop, transferred to the surface of the next solution, and thus passed from solution to solution.

The first step in the technique was to float sections for 20 min on 1% aqueous periodic acid at room temperature. They were rinsed by several brief passages on distilled water baths and a 30-min stay on the last of these baths. The sections were then taken to the silver methenamine solution. This solution was prepared fresh daily. 2 ml of 5% silver nitrate were added drop by drop to 18 cc of 3% hexamethylenetetramine,

followed by 2 ml of 2% sodium borate in distilled water. The sections were floated on this solution in a 60–70°C oven. This was done in a dark room. After 60 min, the lights were briefly turned on to examine the sections. If they showed a yellow tinge, they were taken to distilled water for rinsing. If not, they were kept in the oven for another 20 min, when the yellow tinge usually appeared. After several rinses on distilled water, the last one for over an hour, the sections were left for 5 min on 3% sodium thiosulfate, and rapidly rinsed again on distilled water. The sections were then picked up on formvar-coated grids and examined in a Siemens microscope without counterstaining.

Control sections were also used, which were not treated with periodic acid before being taken to the silver solution.

For comparison with the light microscope pattern, paraffin sections of Carnoy- and Bouin-fixed tissues were stained by PA-silver in a similar manner and were examined in parallel with adjacent sections stained with PA-Schiff (69).

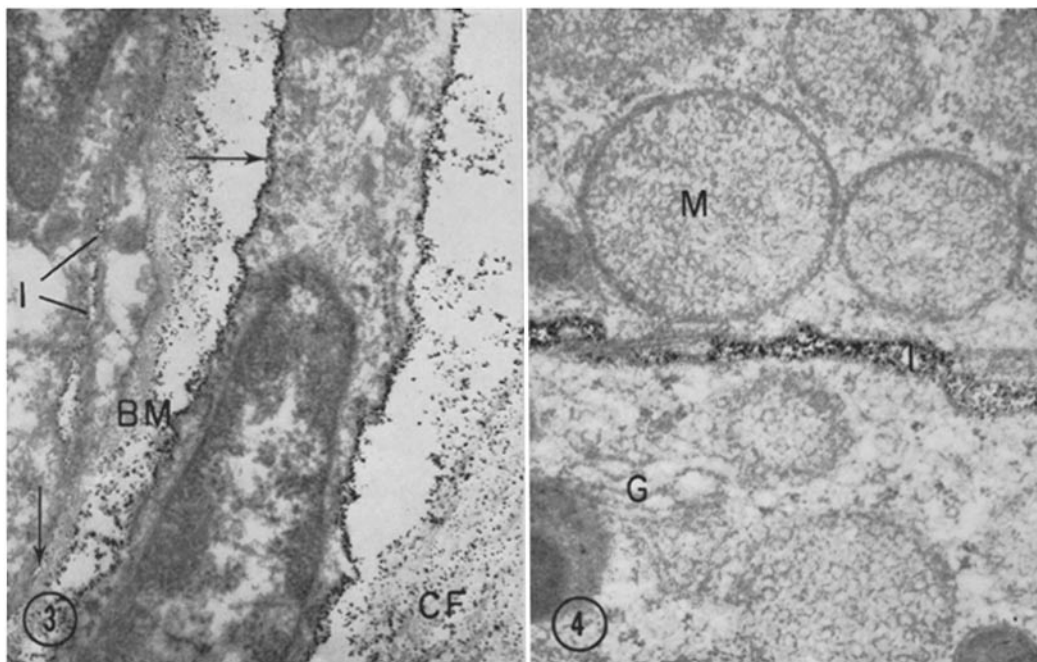


FIGURE 3 *Fibrocyte*. The horizontal arrow points to the accumulation of thorium particles at the surface of a fibrocyte. Colloidal particles are also seen at the surface of collagen fibrils at lower right (*CF*) and in the space (*I*) which separates two endothelial cells. While the basement membrane (*BM*) remains unstained, a few particles are present along the basal surface of the endothelial cells (vertical arrow). $\times 30,000$.

FIGURE 4 *Adrenal cortex*. Particles may be seen in the intercellular space (*I*). *M*, mitochondria; *G*, Golgi apparatus. $\times 38,500$.

RESULTS

Colloidal Thorium

Colloidal thorium is seen at the surface of free cells, such as lymphocytes (Fig. 1, arrow), red blood cells (Fig. 2), and fibrocytes (Fig. 3). A similar accumulation of thorium is evident at the free surface of epithelia (lower arrow in Fig. 5) and endothelia (Fig. 2 and upper arrow in Fig. 5). Where the plasma membrane of the cells is visible, it is clear that the particles of metal are located on its outer surface.

In the interspace between cells, colloidal thorium is usually absent or is present only in small amounts (Figs. 3, 4, and 6, *I*). While basement membranes are always unreactive (Figs. 3, 5, and 6, *BM*), the space separating them from the overlying epithelium or endothelium may contain a fine metal deposit (Fig. 6).

Periodic Acid—Silver Methenamine

In the *light microscope*, the periodic acid—silver methenamine (PA—silver) technique gives results which are somewhat similar to those of the PA—Schiff technique. Thus, both stain mucus (Fig. 7 and 8). The staining of cell surfaces recently described after PA—Schiff staining (98) may also be observed with PA—silver (Figs. 9 to 12).

It is already known that some structures stain with the Schiff reagent in the absence of periodic acid treatment, so that a reaction is said to be specific only if it fails to occur in controls that have not been taken through the acid (61). Similarly, with the PA—silver technique, there are structures that stain even when the periodic acid treatment is omitted.

Hence, for *electron microscopy*, the first step was to examine controls treated with silver methenamine alone. In such controls, chromatin and nucleoli stained (Figs. 13 and 21) and so did the ribosomes in the cytoplasm (Fig. 15). In connective tissue, collagen fibers appeared as dark spots separated by distinct light spaces (Fig. 17). Red blood cells and blood plasma were both uniformly blackened (Fig. 21). Silver was also found in the crystals of eosinophil granules, in various pigments and unidentified granules. Finally, whereas cell membranes did not stain, the desmosomes were often indicated by two bands corresponding to the plates at their intracytoplasmic surfaces (Fig. 19, arrows). In all these sites, the staining was unspecific and, therefore, unrelated to the purpose of this investigation.

Conversely, these structures which stained after periodic acid treatment were taken to be reactive and will now be examined.

BLOOD CELLS

After periodic acid and silver treatment, a reactive line is clearly seen at the surface of those blood cells encountered in connective tissue, as shown by the eosinophil in Fig. 23, and the lymphocytes in Fig. 29 (arrows). Newly formed platelets about to be released from the cytoplasm of megakaryocytes are sharply outlined (Fig. 24). However, no distinct line was seen at the surface of red blood cells (Fig. 22).

CONNECTIVE TISSUE: MUSCLE

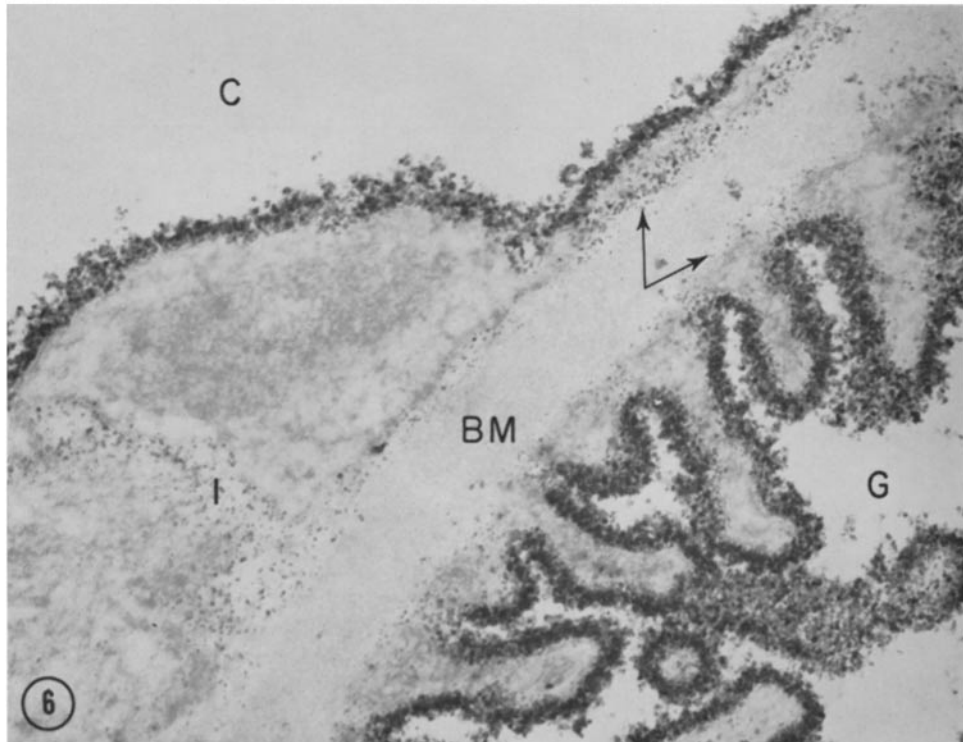
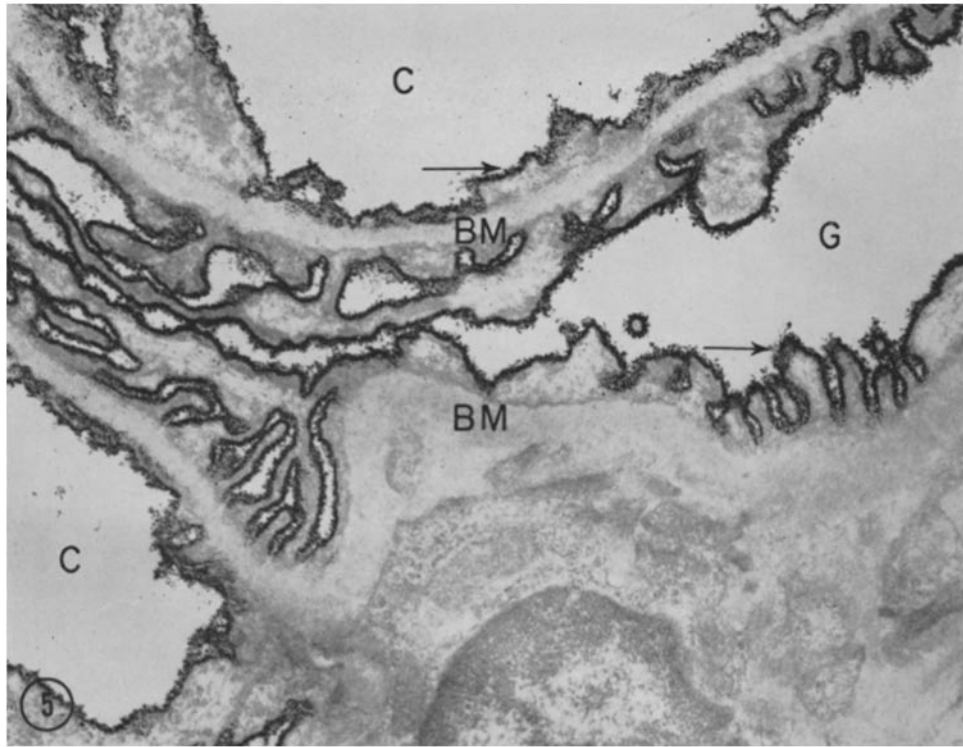
The stained material at the surface of fibrocytes is scanty (Fig. 18) and seems to be continuous

Electron microscope view of kidney glomeruli stained with colloidal thorium.

FIGURE 5 *Glomerulus*. The free surface of the endothelial cells lining the two capillaries (*C*) and the surface of the podocyte processes lining the glomerular space (*G*) are heavily coated with thorium particles (arrows). The basement membrane (*BM*) remains unstained. $\times 21,500$.

FIGURE 6 *Glomerulus*. At this high magnification, it is possible to see not only the thick layer of thorium particles at the free surface of the capillary endothelium (*C*) and of the podocytes lining the glomerular space (*G*), but also a light scatter of particles along the basal surface of endothelial cells (vertical arrow) and in the light space which separates the unstained basement membrane (*BM*) from the feet of the podocytes (oblique arrow). They are also present in the intercellular space (*I*) between two endothelial cells. $\times 77,000$.

Light microscope comparison of (left) PA—Schiff— and (right) PA—Silver—stained preparations.



with intercellular material. Next to these cells, there are often collagen fibers, which not only display the unspecific staining mentioned above (Fig. 17) but are embedded in hazy material which does stain specifically (Fig. 18). Reticular cells (Figs. 23 and 25, *Re*) and mast cells (Fig. 29, lower right corner) clearly show a surface reaction. Similarly, in the tooth, the odontoblasts exhibit specific staining at their interfaces (Fig. 16).

Striated and smooth muscle fibers are enclosed within heavily reactive lines (Fig. 23). In the arteriole represented in Fig. 22, the reactive band surrounding smooth muscle fibers (*Mu*, oblique arrows) merges with the basement membrane of the endothelium. Within the merged membranes, there are unstained spaces believed to contain elastic fibers (*EF*).

EPITHELIA

Taking the intestinal epithelium as an example, the surface of the microvilli at the cell apex shows strongly reactive material (Figs. 26 and 27). Lateral membranes are associated with a moderate reaction (Figs. 26 and 28, arrows). The basal surface is lined with a heavily stained basement membrane (Fig. 29, *BM*), but the 500-A space seen in normally stained preparations between plasma membrane and basement membrane is not distinguishable. In any case, stained material sur-

rounds the cell, except where the lateral membrane is about to reach the surface, that is, in the region corresponding to the tight junction of the terminal bar (Figs. 26 and 27, *tj*). On the other hand, the region of the wide junction is stained, often more than the rest of the interspace.

The endothelial cells lining blood vessels are also enclosed within stained lines (Fig. 22).

The ameloblasts of the enamel organ are separated by a discontinuous stained line (Figs. 14 and 32). The interruptions may correspond to regions where adjacent cell membranes fuse (tight junctions). At the base of the cells, the interface with the cells of the stratum intermedium appears as a convoluted reactive line, which continues into the middle plate of desmosomes (Fig. 20).

EXOCRINE GLANDS: PANCREAS

The contours of acinar pancreatic cells, including those of the apical microvilli, are delineated by the stain (Figs. 30 and 31). The region corresponding to the tight junction of terminal bars remains unstained (Figs. 30 and 31, *tj*), though there are exceptions (Fig. 31, arrow).

ENDOCRINE GLANDS: PITUITARY, THYROID

Lines moderately stained with PA-silver enclose the various types of endocrine cells, such as those of the pituitary (Fig. 36, arrows) and thyroid

FIGURE 7 *Sublingual gland*. Carnoy fixation. PA-Schiff stain. Mucus is intensely stained (*M*). In the duct, the vertical arrow points to positive material at the free surface. Basement membranes are indicated by greyish lines (*BM*). $\times 650$.

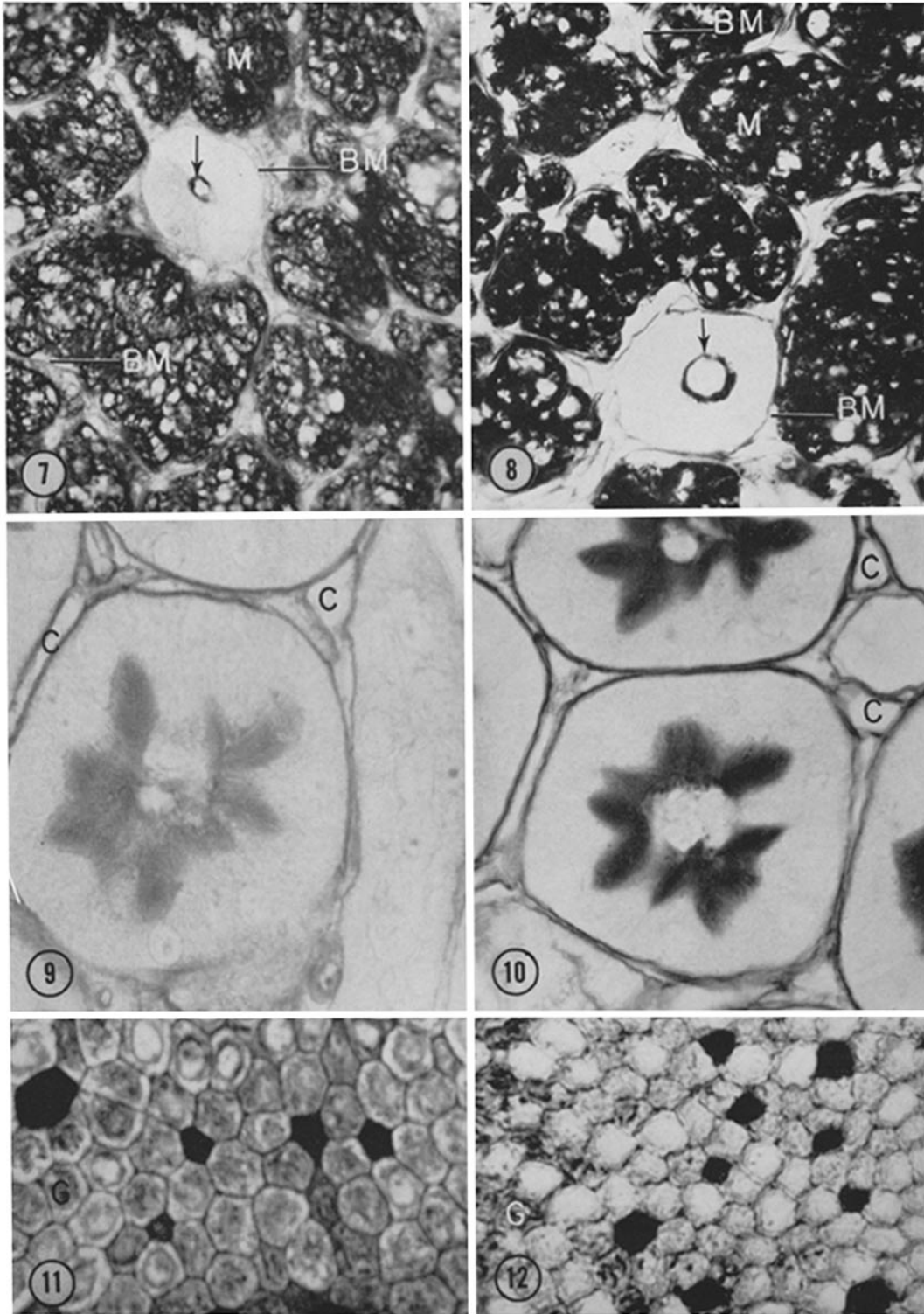
FIGURE 8 *Sublingual gland*. Carnoy fixation. PA-silver stain. Same pattern as in Fig. 7. $\times 650$.

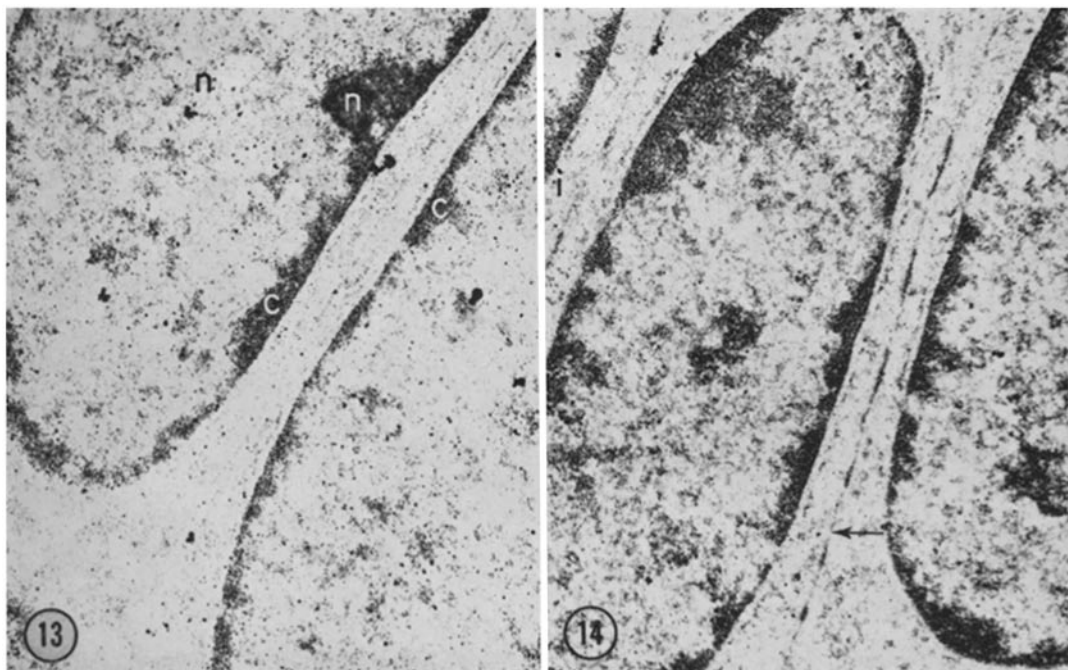
FIGURE 9 *Proximal convoluted tubule of kidney*. Carnoy fixation. PA-Schiff stain. Brush border and basement membrane are intensely stained. *C*, blood capillary. (The lateral membranes of the cells are too irregular to be seen in sections.) $\times 1300$.

FIGURE 10 *Proximal convoluted tubule of kidney*. Carnoy fixation. PA-silver stain. As with PA-Schiff stain, brush border and basement membrane stand out. $\times 1300$.

FIGURE 11 *Cross-section of the cells of intestinal epithelium*. Bouin fixation. PA-Schiff-toluidine blue stain. The dark masses in this figure are PA-Schiff-stained goblet cells. From them, lines extend radially which are the stained lateral surfaces of columnar cells. In the left part of the picture, the section cuts through the nuclei of epithelial cells and through moderately stained Golgi elements (*G*). $\times 1300$.

FIGURE 12 *Cross-section of the cells of intestinal epithelium*. Carnoy fixation. PA-silver stain. Goblet cells may be identified as dark masses from which the lateral surfaces of columnar cells radiate. In the left part of the picture, the cells contain intensely stained Golgi elements (*G*). $\times 1300$.





Tooth. Control and PA-silver-stained sections.

FIGURE 13 *Ameloblasts*. Control. Chromatin (*c*) and nucleolus (*n*) are stained in the nuclei. The intercellular space remains unstained. $\times 17,500$.

FIGURE 14 *Ameloblasts*. PA-silver stain. As compared with the control, staining of nuclei appears to be slightly increased. The intercellular space appears as an interrupted line, along which interruptions are thought to correspond to regions of membrane apposition (horizontal arrow). $\times 16,500$.

gland (Figs. 33–35). Again, the reactive lines separating thyroid follicular cells are usually interrupted at the region of the tight junction of the terminal bars (Fig. 35, *tj*), whereas the wide junction shows slightly increased staining (Fig. 33). Furthermore, no clearcut line separates the deeply reactive luminal colloid (*C*) from the microvilli at the surface of follicular cells (Fig. 35).

KIDNEY

In the proximal convoluted tubules, strongly reactive material is located between the microvilli of the brush border (Fig. 37, *bb*) and may be followed in apical invaginations of the plasma membrane (Figs. 37 and 38, *Inv*). In distal convoluted tubules, the well developed basal membrane infoldings appear as dark lines against an otherwise unstained cytoplasm (Fig. 39, arrows).

NERVOUS SYSTEM: CEREBRAL CORTEX

Nerve cells are outlined by a discontinuous reactive line, which may be traced along dendrites and axons (Fig. 42). In the neuropile, nerve and glial processes are surrounded by thin reactive lines (Fig. 42). The various lines may present thickenings and interruptions. Faintly stained groups of small vesicles are next to some of the thickenings which, therefore, correspond to synaptic clefts (Figs. 40 and 41).

DISCUSSION

Staining with Colloidal Thorium

In light microscopy, the colloidal iron-Prussian blue technique outlined the entire surface of cells (98). In contrast, only the free surface of cells was regularly stained with the colloidal thorium used

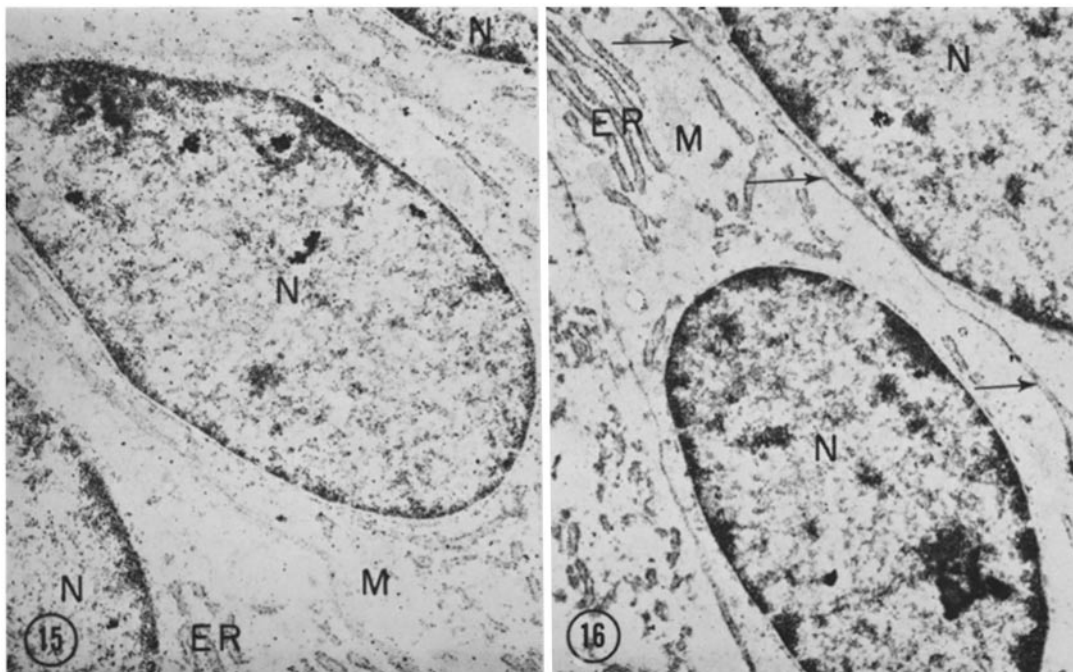


FIGURE 15 *Odontoblasts*. Control. In addition to the nuclei (*N*), ergastoplasm (*ER*) may be seen in the lower part of the picture. A dense material fills the sacs of the rough endoplasmic reticulum whose membranes are studded with dark dots which are the ribosomes. Under a barely visible mitochondrion, the letter *M* may be seen. $\times 12,000$.

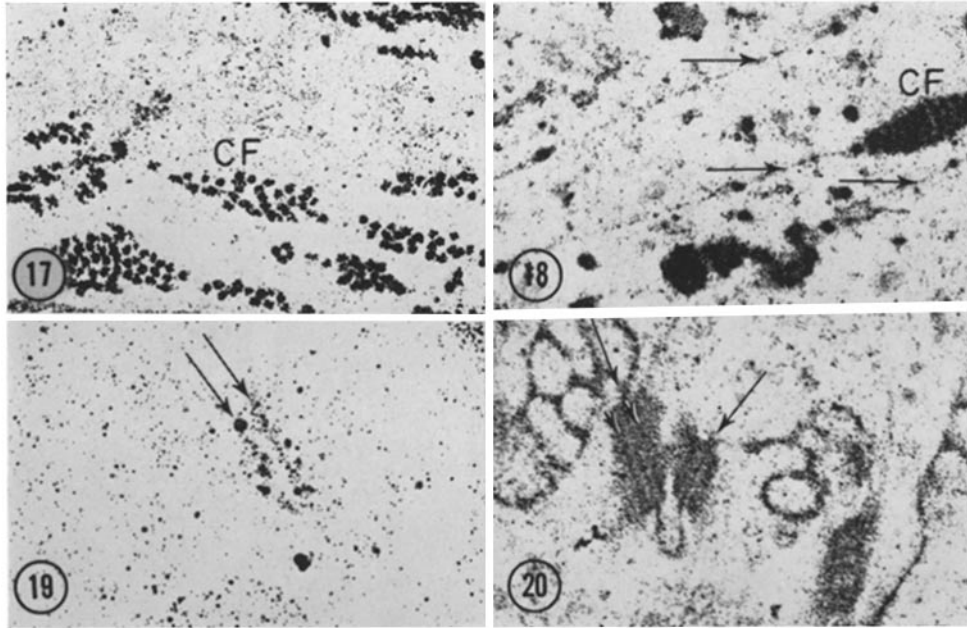
FIGURE 16 *Odontoblasts*. PA-silver stain. As compared with Fig. 15, the general contrast is slightly increased and the cell surface (horizontal arrows) becomes intensely stained. *N*, nucleus; *ER*, ergastoplasm; *M*, mitochondria. $\times 12,000$.

for electron microscopy (Figs. 1-3, 5, and 6). There, thorium particles were located outside the plasma membrane, which the uranyl acetate counterstain showed as a thin black line. Thorium particles were rare between cells (Fig. 4) and absent inside cells (in contrast with light microscopy results which showed frequent Golgi staining; references 2, 98). Hence, when the specimens had been placed in the solution of colloidal thorium, penetration must have been poor. Thus, the technique was not quite satisfactory. An attempt was made to overcome the difficulty by staining methacrylate sections with colloidal iron (26) or colloidal thorium (99) but, again, results were poor.

Even the localization of particles at the cell surface (Figs. 1-3) might be questioned, since the material could be adsorbed unspecifically. However, since colloidal iron staining of the cell surface

for electron microscope study may be abolished by methylation, as shown with intestinal cells (26), or by neuraminidase treatment, as shown with ascites tumor cells (40), it is likely that the staining is not an artefact but is due to the presence of material reacting with colloidal thorium.

The blockage of the staining by methylation (26) indicated that the reactive material carried acidic residues, such as carboxyl or sulfate groups. Indeed, there is good evidence that, when cationic acid stains such as colloidal iron and thorium are used at pH 2.5, they are selectively bound to acidic groups (85, 99). The staining of the interspaces between intestinal cells with Alcian blue at pH 3 (114) also pointed to acidic groups at the cell surface. Now the acidic groups free at pHs 2.5-3 could be in nucleic acids or in carbohydrates. Since by lowering the pH the staining of nuclei disappeared but that of the cell surface persisted,



Electron microscope view of various tissues. Control and PA-silver-stained sections.

FIGURE 17 *Connective tissue*. Control. Collagen fibrils (*CF*) appears as dark spots separated by empty spaces. $\times 20,000$.

FIGURE 18 *Connective tissue*. PA-silver stain. The collagen fibrils (*CF*) are now embedded in a dense material. The horizontal arrows point to the cell surface of fibroblasts lying side by side. $\times 20,000$.

FIGURE 19 *Stratum intermedium of incisor tooth*. Control. The two irregular black bands indicated by small arrows are thought to represent the two cytoplasmic plates of a desmosome. $\times 27,500$.

FIGURE 20 *Stratum intermedium of tooth*. PA-silver stain. The convoluted line separates the base of an ameloblast (above) from a cell of the stratum intermedium (below). At the long arrows, the intermediate plate of the desmosomes is seen to be continuous with the stained intercellular line. The two small arrows point to the cytoplasmic plates (more deeply stained than in Fig. 23). $\times 27,500$.

it was likely that carbohydrates rather than nucleic acids were involved. Since, furthermore, treatment with sialidase abolished the surface staining of ascites tumor cells (40) and ova (109), the carbohydrates must contain sialic acids. Incidentally, we found that hyaluronidase had no effect on the staining of the cell surface with colloidal iron, so that the role of hyaluronic acid postulated in the past (27) need not be considered further. It was concluded that a material rich in acidic groups, probably sialic acid groups, is present at the outer surface of cells.

Staining with PA-Silver

Since the staining of the cell surface observed in light microscopy with the PA-Schiff reaction

(Figs. 9 and 11) was usually duplicated with PA-silver (Figs. 10 and 12), PA-silver was used in electron microscopy to examine the fine detail of the stained material at the cell surface. However, the specificity of the reaction had to be critically examined.

SPECIFICITY OF PA-SILVER REACTION

The reaction is carried out in two steps: (a) *periodic acid oxidizes* 1,2-glycol and alpha-amino groups (such as are found in glycoproteins), thereby transforming them into aldehydic groups (43, 95); (b) *these aldehydic groups reduce* the silver tetramine contained in the methenamine silver reagent, with release of free silver (as in Tollen's test; references 65, 82).



FIGURE 21 *Cross-section of an arteriole. Control. Between the nuclei (N_1 , N_2) of two endothelial cells, the lumen contains a red blood cell (RBC) and blood plasma (Bp). The fusiform nucleus (Nm) of a smooth muscle fiber is visible. Collagen fibrils present in the adventitial layer of the arteriole appear at upper left. $\times 24,500$. Compare to Figure 22.*

In spite of this sound chemical basis, the PA-silver reaction has been viewed with considerable skepticism (64). On the basis of our experience, we shall review the major difficulties encountered and suggest how they may be overcome:

1. *Silver deposition may occur in the absence of periodic acid oxidation.* Reducing groups might be present in tissue sections and cause silver deposition, regardless of whether or not the sections had first been oxidized with periodic acid. Such unspecific silver deposition was detected by examination of control sections stained with the methenamine silver solution without passage through periodic acid. The sites were found to be those containing nucleic acids (chromosomes, nucleolus, ribosomes; Figs. 13 and 15), collagen fibers (Fig. 17), plasma and red blood cells (Fig. 21), some pigments, mast cell granules, etc. Some of these unspecific reactions might be due to preexisting aldehydic groups. Thus, according to Lhotka (63), blockage of alde-

hydes suppressed the staining of collagen fibers with silver. Reducing groups other than aldehydes may also be involved; thus, the unspecific staining of pigments rich in melanine may be due to the reducing properties of this substance (80).

2. *Fixation may modify the extent of the staining.* When tissues are preserved in glutaraldehyde or other aldehyde-containing fixatives, some structures might adsorb the aldehyde and then reduce the silver reagent. In our experience, fixation in a glutaraldehyde-formaldehyde mixture (55) increased the number of structures unspecifically stained. It has also been said that silver is taken up by histones after formaldehyde fixation (8-10), a process which may account for the unspecific staining of chromatin.

Osmium tetroxide has been commonly used to fix tissues prior to staining with PA-silver (47, 76, 78, 84, 110). However, a number of structures, particularly membranes, were stained unspecifically.

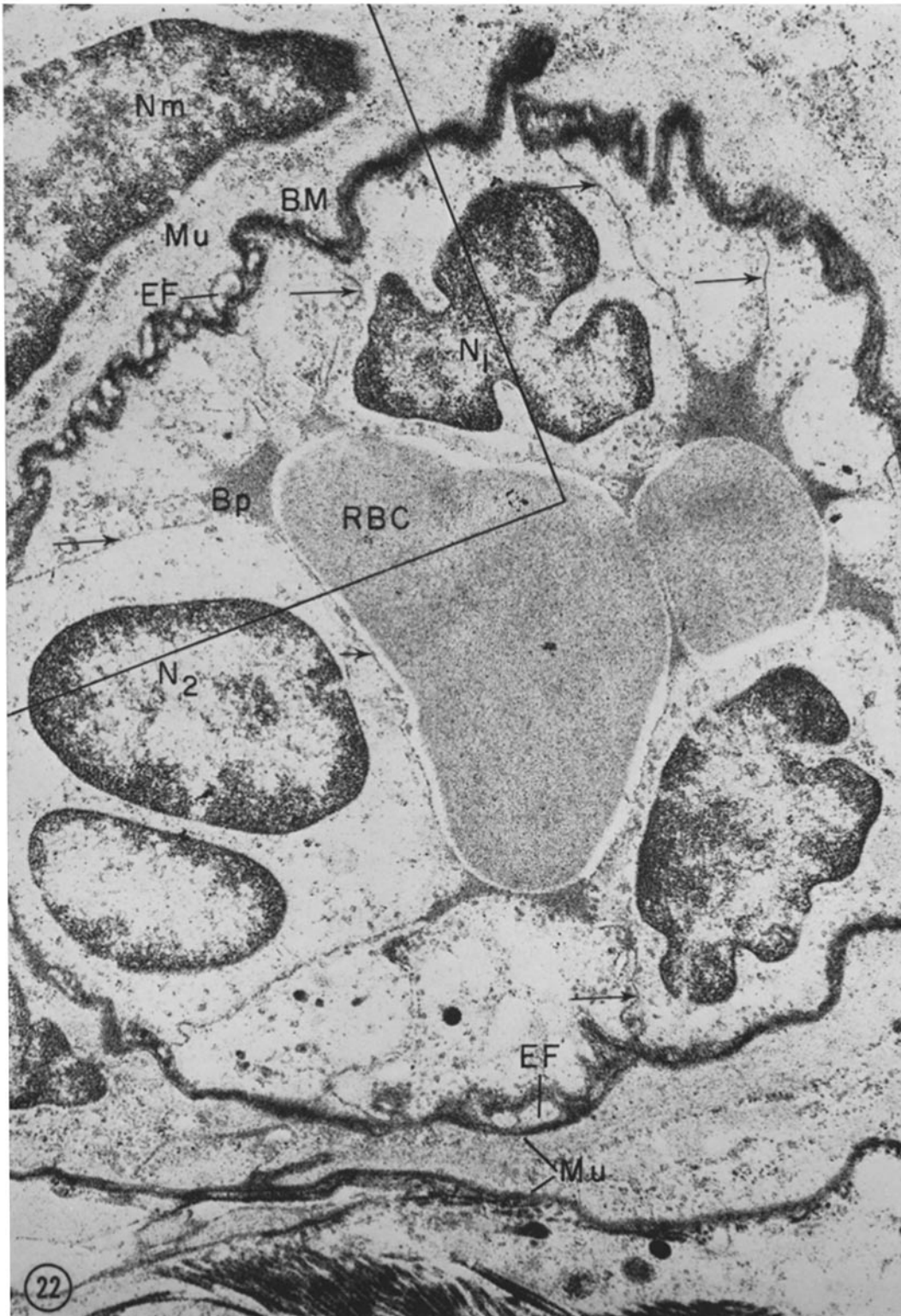
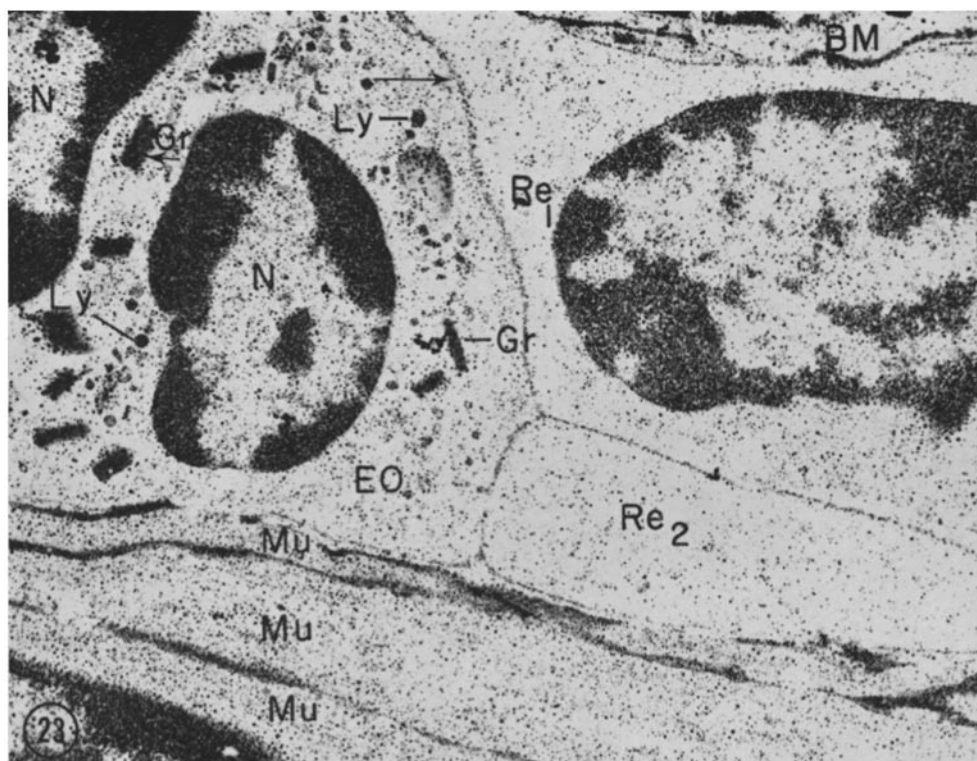


FIGURE 22 *Electron microscope view of arteriole.* PA-silver stain. The field outlined in black corresponds to the one presented in Fig. 21. In addition to unspecifically stained nuclei (N_1 , N_2 , N_m), red blood cells (RBC), blood plasma (Bp), and collagen fibrils (*lower edge*), there is specific staining of cell surfaces (horizontal arrows) and of the basement membranes (BM) separating smooth muscle fibers (Mu) from endothelial cells. In the basement membrane, holes may be seen which probably correspond to unstained elastic fibers (EF). $\times 15,000$.



Free cells. PA-silver stain.

FIGURE 23 Core of intestinal villus. At upper left, an eosinophil (*Eo*) contains the unstained nucleus (*N*) and crystals of eosinophilic granules (*Gr*), and the specifically stained lysosomes (*Ly*). At right, two reticular cells (*Re*₁, *Re*₂) are separated from each other and from the eosinophil by a specifically stained line. At top, the reticular cell (*Re*₁) is separated from an intestinal epithelial cell by the basement membrane (*BM*). The lower part of the picture is occupied by three longitudinally cut smooth muscle fibers (*Mu*) surrounded by heavily stained lines. $\times 19,000$.

cally after such fixation. Perhaps, osmium tetroxide derivatives were bound to these membranes and could then reduce the silver solution. In any case, the results with osmium tetroxide have been poor, so much so that the use of the PA-silver reaction in electron microscopy has been discredited.

Of the fixatives examined, glutaraldehyde yielded a relatively small number of unspecific reactions and was adopted for this investigation.

3. *The lack of stability of silver solutions*, especially at 60°C, may cause artefactual precipitates. Fine silver deposits appeared not only on the walls of the Petri dish containing the solution, but also on the sections, thus causing the "background" seen in the figures. This background was minimized by preparing fresh solutions daily and by leaving the sections in the methenamine silver bath for no more than 60–80 min.

Under these conditions, any reaction observed in sections given the complete PA-silver treatment but absent in control sections was considered as being specific and attributable to the presence of 1,2-glycol and/or alpha-amino alcohol groups. Such specific reactions were observed in a series of materials: mucus (Fig. 8), thyroid colloid (Figs. 33 to 35), basement membrane (Figs. 30, 34, 39), lysosomes (Fig. 33), Golgi saccules (Fig. 32), cartilage matrix, etc. The main observation was the presence of specifically stained material at the surface of most cells (Figs. 14, 16, 18, 22, and following).

It was stated above that PA-silver, like PA-Schiff, is specific for 1,2-glycol and alpha-amino alcohol groups, such as are found in glycogen and glycoproteins (43, 62). With the PA-silver technique used here, glycogen did not react. It was

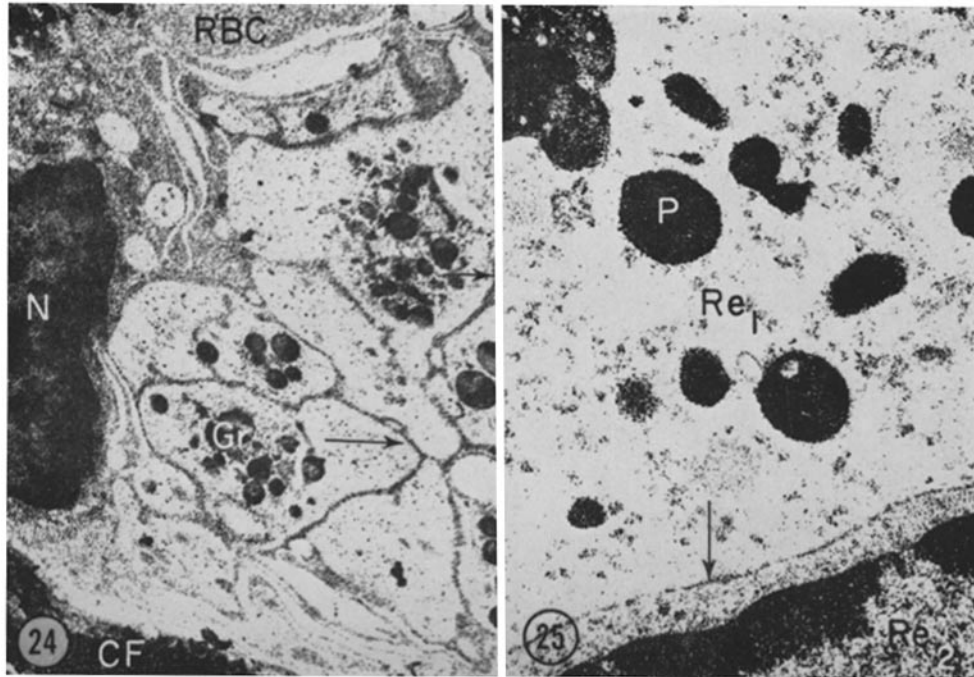


FIGURE 24 *Megakaryocyte in red pulp of spleen.* The nucleus of the megakaryocyte is visible at left (N). Dark lines (arrows) divide the cytoplasm into small areas containing specifically stained granules (Gr). Each of these areas is thought to correspond to a platelet whose surface is delineated by a newly formed cell coat. At top, a red blood cell (RBC) may be seen. Collagen fibrils (CF) are present at lower left. $\times 14,000$.

FIGURE 25 *Spleen reticular cells.* The stained line indicated by an arrow separates two reticular cells (Re_1 , Re_2). The cytoplasm of the reticular cell in the upper part of the picture contains pigment granules (P). $\times 22,500$.

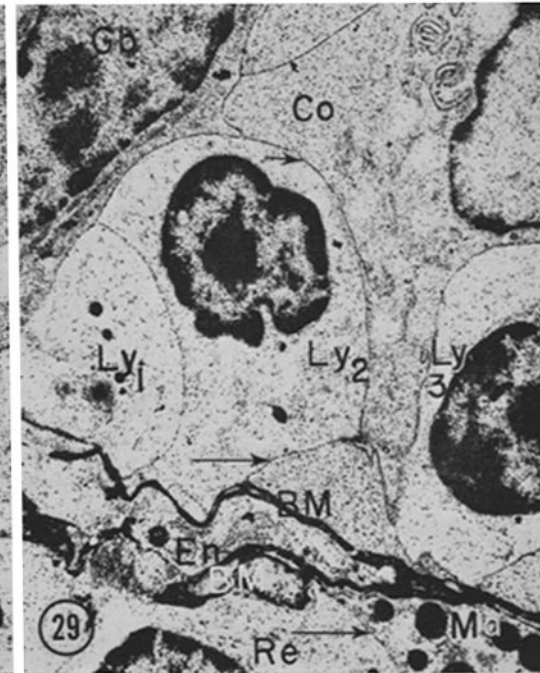
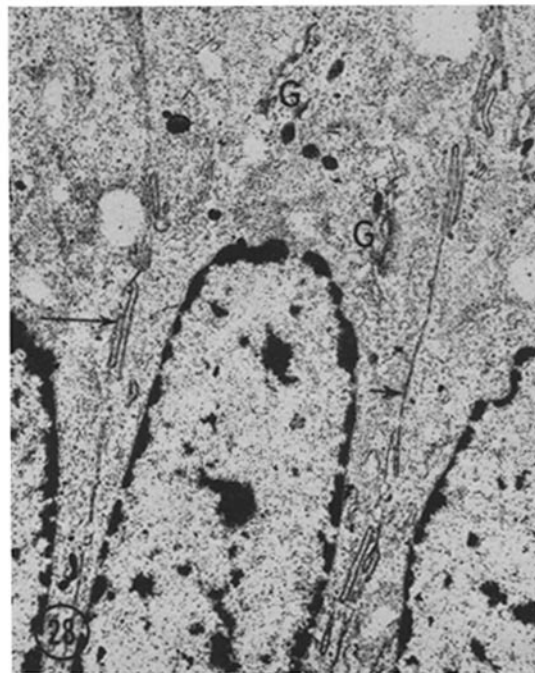
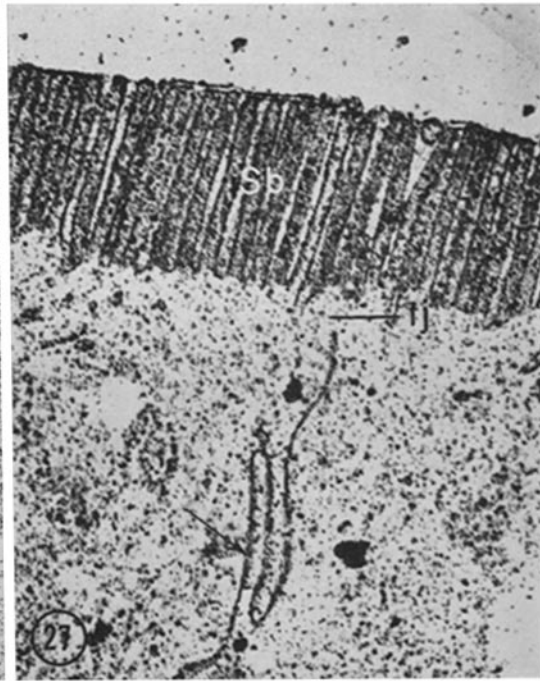
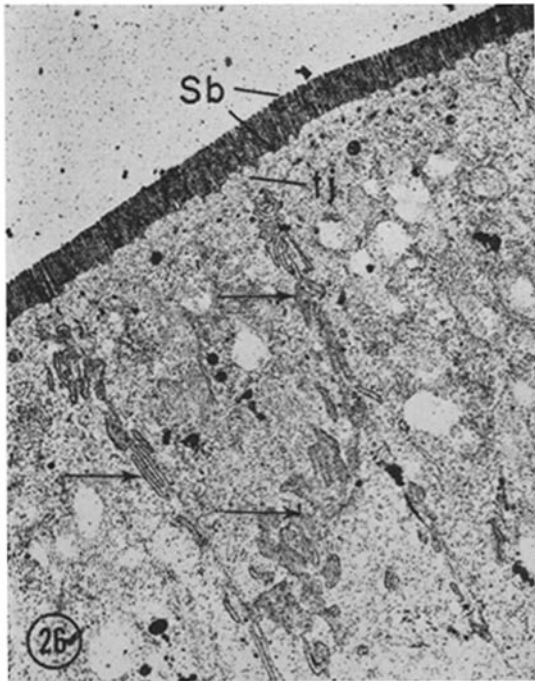
Small intestine. PA-silver stain.

FIGURE 26 *Apical region of epithelial cells.* The striated border (Sb) at the apex of the three epithelial cells is intensely stained. The highly convoluted intercellular space may be followed at arrows. The tight junctions remain unstained (tj). $\times 5200$.

FIGURE 27 *Striated border of epithelial cells.* At this higher magnification, the lack of staining of the tight junction (tj) of the terminal bar is clearcut. $\times 25,500$.

FIGURE 28 *Nucleus and supranuclear region of epithelial cells.* The lateral surfaces of the cell are indicated by arrows. In the supra-nuclear region, Golgi elements and associated granules are specifically stained (G). $\times 8250$.

FIGURE 29 *Base of epithelial cells.* At top, the lower portions of a goblet cell (Gb) and columnar cells (Co) are seen. Three lymphocytes (Ly) are in the center of the picture. All these cells are separated from each other by stained lines (arrows). In the lower part of the picture, a tangentially sectioned endothelial cell (En) may be seen between the two heavily stained basement membranes (BM). The lowest part of the figures depicts a reticular cell (Re) and a mast cell (Ma) in the core of the villus. $\times 8250$.



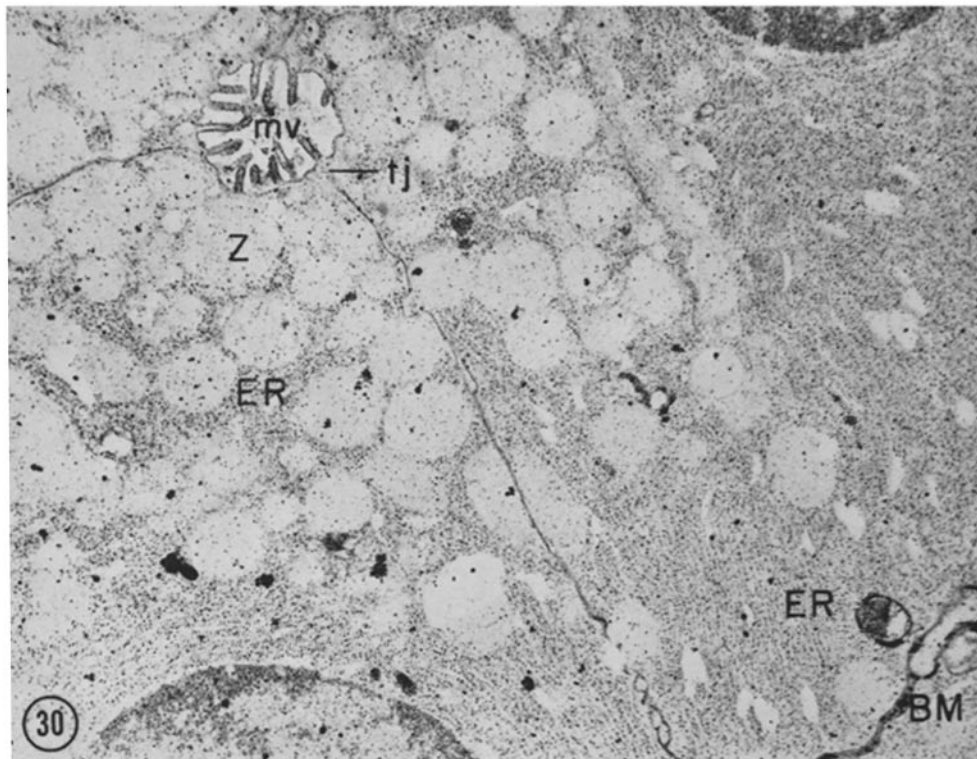
tentatively, concluded that a material rich in glycoprotein is present at the surface of cells.

FINE LOCALIZATION OF PA-SILVER-STAINED MATERIAL AT THE CELL SURFACE

A single stained line was observed at the interface of epithelial cells (e.g. Figs. 27 and 28), between adjacent free cells (Fig. 29), and even at membrane infoldings (Fig. 39). In fact, nearly all cells examined had a stained line at the surface. The exceptions were few. One was the red blood cells (Fig. 22). Either stainable material was actually lacking at the surface or its presence was obscured by the unspecific staining of cell substance and surrounding plasma (Fig. 21). (In contrast, the surface staining of red blood cells with colloidal thorium was unambiguous; Fig. 2.) Around other free cells, such as lymphocytes, the

presence of a stained layer was sometimes doubtful but was always definite when free cells were closely packed (Figs. 23 and 29). Around connective tissue cells (Fig. 18), the stained layer often appeared as an irregular thin line, poorly distinguishable from the ground substance; presumably, stained material was present but, because of the wavy outline of the cells, was not readily distinguishable. At the free surface of thyroid cells (Fig. 33), no distinct line was seen along the microvilli, perhaps because the line blends into the stained colloid. As a matter of fact, the whole colloid might be an expanded cell coat.

The thickness of the stained lines seen between adjacent cells in the electron microscope was measured by using fields illustrated in this article but at higher magnifications (20,000, 40,000 and



PA-silver stain.

FIGURE 30 *Pancreatic acinus*. The basement membrane of an acinar cell is visible at lower right (*BM*). The stained intercellular space in center may be followed to the tight junction (*tj*), which remains unstained. Staining is slightly enhanced at the surface of microvilli (*mv*). *ER*, ergastoplasm; *Z*, zymogen granules. $\times 14,500$.

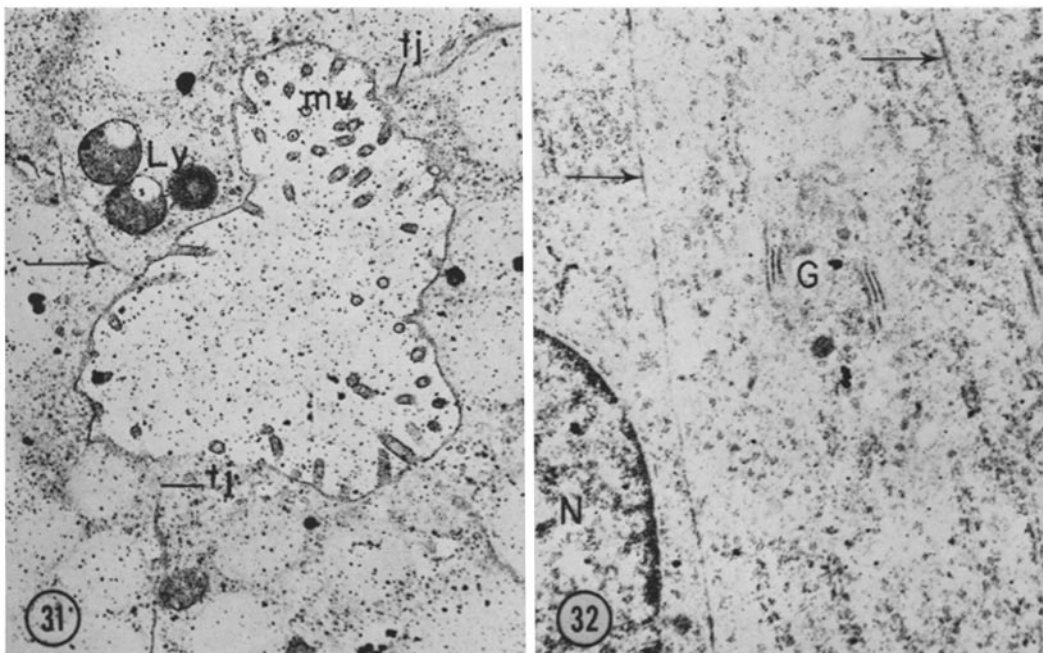


FIGURE 31 *Lumen of a pancreatic acinus.* The staining of the intercellular spaces at lower left and upper right stops at the level of the tight junctions (*tj*). At upper left, near a group of lysosomes (*Ly*), the staining may be followed up to the lumen (arrow). Staining is enhanced at the surface of microvilli (*mv*). $\times 22,500$.

FIGURE 32 *Golgi region in ameloblast.* The nucleus of an ameloblast (*N*) is visible at lower left. Two stained intercellular spaces are indicated by horizontal arrows. Specifically stained material fills the flattened saccules of the Golgi apparatus seen on either side of the letter *G*. $\times 20,000$.

70,000). The average of 34 measurements gave a mean thickness of 140 Å.

In what part of the membrane complex was the stained material located? It might be either in the plasma membrane or outside it. If only the plasma membrane were stained, double rather than single lines would be expected between cells. Furthermore, at the basal surface of epithelial cells, the 500-Å space separating the plasma membrane from the stained basement membrane (37) should show up as it does in ordinary electron microscope preparations. Since neither of these conditions was fulfilled, there must be stained material outside the plasma membrane.

However, it was possible that both plasma membrane and outer material were stained. The space between epithelial cells measures 110–150 Å and each one of the two adjacent plasma membranes is 60–100 Å thick (27). Hence, staining of all these structures should yield a 230–400 Å-thick line.

This was definitely more than the 140 Å lines measured between cells. Presumably then, the plasma membrane did not stain.

There remained the possibility that, besides the interspace material, the outer leaflet of the plasma membrane was also stained. However, at the tight junction of terminal bars (zona occludens), the staining usually vanished (Figs. 27 and 35). Since the outer leaflets of the plasma membranes are fused in this region and the interspace disappears, the outer leaflets did not seem to be stained. Even in the few cases of weak spotty staining of tight junctions (Fig. 31), the possibility of outer leaflet staining was less likely than the persistence of small amounts of interspace material (since it is known that some junctions may not be quite "tight," as observed in pancreas, reference 107; and gastric mucosa, reference 35). In conclusion then, the stained material is likely to be external to the plasma membrane.

Examination of the cell surface in conventional electron microscope preparations has shown filaments attached to the plasma membrane of many cells (7, 12-14, 16, 17, 21, 37-39, 51, 52, 58, 67, 68, 94, 102, 103, 124), or rows of globular units attached to the plasma membrane of liver cells (Fig. 1 in reference 5). Such filaments and/or globules may well be a component of our stained layer, which would then be tightly attached to the outer surface of the plasma membrane.

The *over-all conclusion so far is the existence of an extraneous layer covering the plasma membrane*, as indicated by the results with PA-silver as well as colloidal iron. Several terms have been utilized to describe this layer: "surface coat," a term used mainly to describe material covering the free surface of epithelial cells (51); "glycocalix," a word referring to a hypothetical extraneous layer which would include the basement membrane (7) and its extensions between cells (15); and "cell coat," a term used to describe a stained layer believed to cover ascites cells (41). The layer which we have just described at the surface of rat cells stains exactly like that on ascites cells (but unlike basement membranes). This layer was, therefore, termed "cell coat" (98).

Nature of the Cell Coat

It was shown above that the staining with colloidal thorium was due to carbohydrate(s) rich

in acidic groups, and the staining with PA-silver, to glycoprotein(s). Hence, the cell coat must contain both acidic groups and glycoprotein(s). It is tempting to think that the acidic groups—presumably sialic acid groups—are carried by the glycoprotein(s).

Indirect evidence for the presence of carbohydrate in cell coat arose from Pease's observation (96) that phosphotungstic acid stains the same material as the PA-Schiff technique, that is, goblet cell mucus, basement membranes, brush border, and cartilage matrix. And, even though he failed to notice the staining of the cell surface with the PA-Schiff reaction, he reasoned that, since phosphotungstic acid stains this cell surface, there must be a "polysaccharide" layer there.

Important conclusions as to the chemical composition of the cell coat were based on a centrifugation technique whereby a rather pure cell membrane fraction was separated from microsomes by equilibration in a sucrose-polymer gradient (120). In this fraction, Wallach and Eylar (118) identified sialic acid. Furthermore, when live cells were treated with sialidase and the membrane fraction was separated, the cells survived even though 98% of the sialic acid of this fraction was lost (118). This result confirmed the presence of sialic acid at the cell surface. It may be added that the sialidase treatment caused no change in the respiratory and glycolytic activity of the cells—a

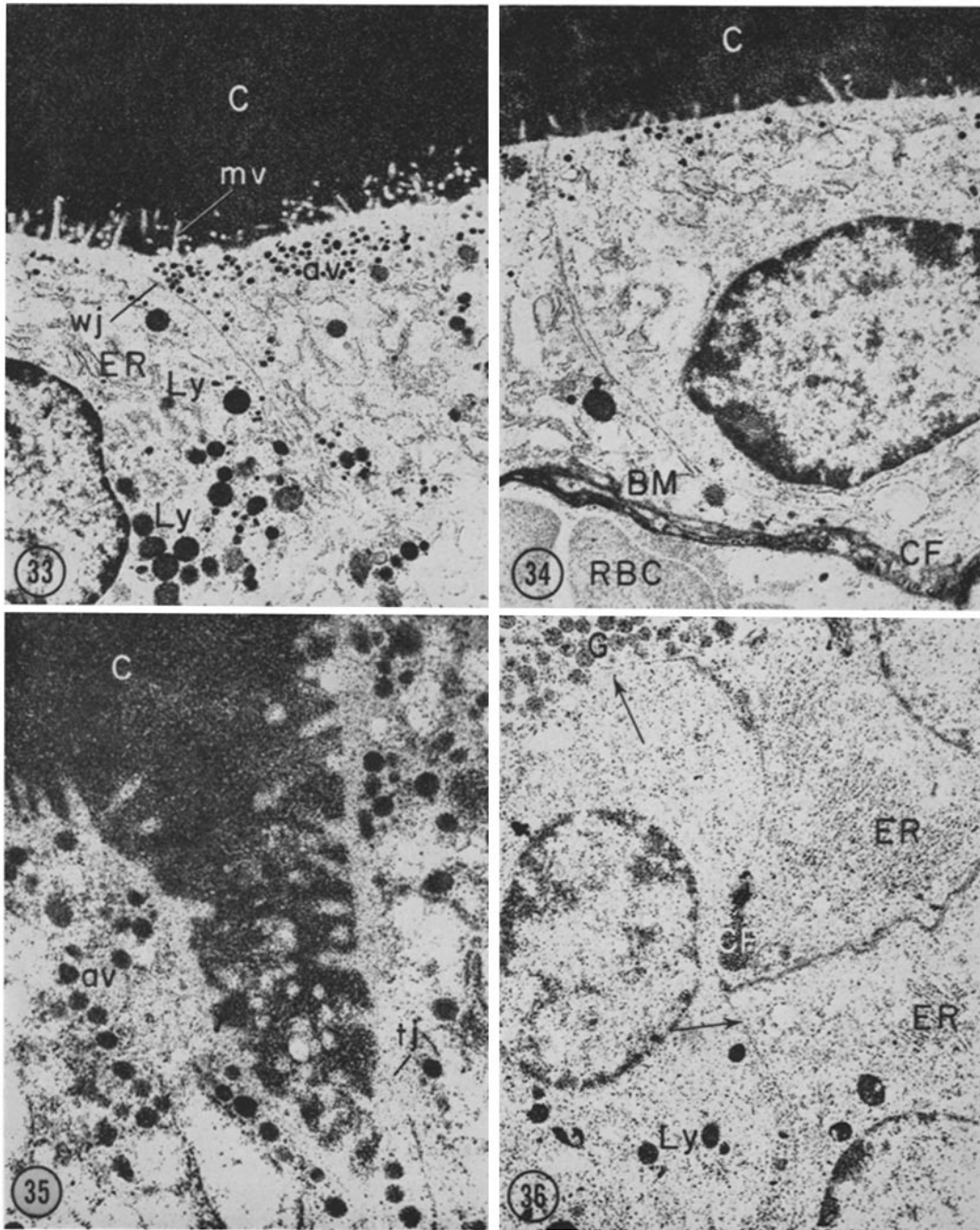
Thyroid. PA-silver stain.

FIGURE 33 *Follicular cells*. At top, the intensely reactive colloid may be seen (*C*). Below the microvilli (*mv*), a light band corresponds to the terminal web. Two kinds of stained granules are seen in the cytoplasm: small apical ones (*av*) close to the apical surface, and larger ones believed to be lysosomes (*Ly*). Staining of the intercellular space is slightly increased in the region of the wide junction (*wj*) of the terminal bar and disappears at the tight junction. The walls of the cisternae of ergastoplasm (*ER*) are studded with unspecifically stained ribosomes. $\times 3000$.

FIGURE 34 *Follicular cells*. In addition to the structures depicted in Fig. 37, the basement membrane (*BM*) of the thyroid follicle may be seen in the lower part of the picture. Cross-sections of collagen fibrils (*CF*) are present in the connective tissue space separating the basement membrane of the follicle from the basement membrane of the underlying capillary containing red blood cells (*RBC*). $\times 8200$.

FIGURE 35 *Apex of follicular cells*. The colloid (*C*) and, in the pale cytoplasm, the apical vesicles (*av*) are stained. The tight junction of the terminal bar (*tj*) remains unstained. $\times 24,000$.

FIGURE 36 *Pituitary gland*. Reactive lines indicated by arrows separate the cells. Some specifically stained granules are seen in the cytoplasm of the cell at upper left. *CF*, collagen fibrils; *ER*, ergastoplasm; *Ly*, lysosomes. $\times 11,000$.



result indicating that removal of sialic acid occurred without damage to the plasma membrane; presumably then, sialic acid was part of an extraneous cell coat rather than of the plasma membrane itself (34, 79, 86).

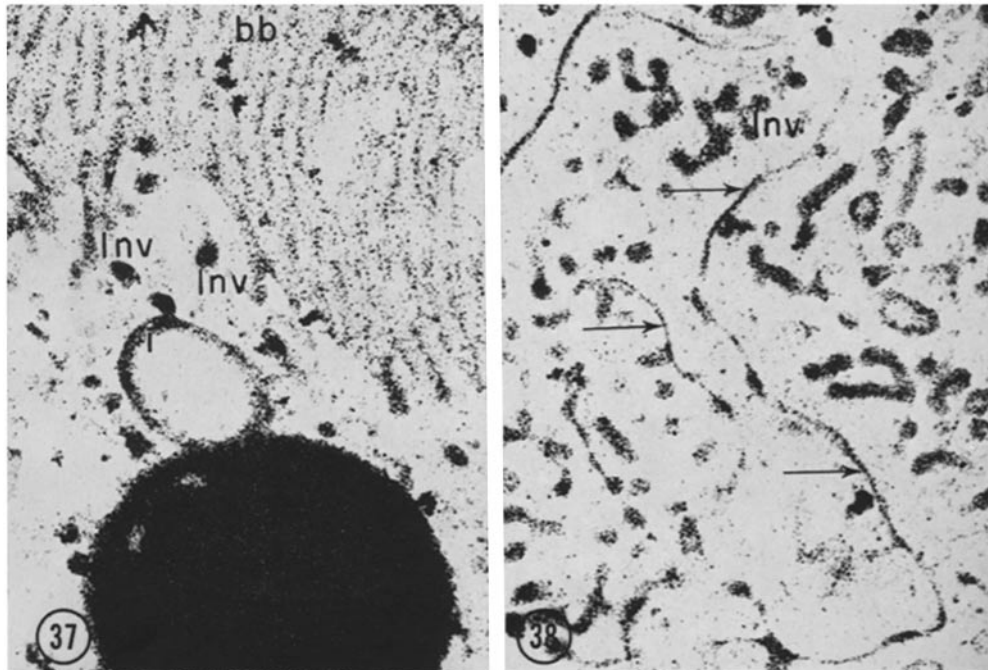
Besides sialic acid, other carbohydrate residues have been identified in the cell coat. Indeed, trypsin, which does not seem to affect the plasma membrane itself, (105) removed from the surface of red blood cells a glycoprotein containing galactose and galactosamine besides sialic acid residues (23, 33). Similarly, a sialomucoprotein rich in galactosamine and sialic acid residues was isolated from the surface of Ehrlich ascites tumor cells (24, 32, 60). Finally, chemical analysis of a membrane fraction from liver cells showed the existence of a glycoprotein with hexose, hexosamine, and sialic acid residues (31). These results

are in line with our histochemical conclusion that the cell coat contains glycoprotein(s) and acidic groups.

PROPERTIES OF THE CELL COAT

So far, the existence of an extraneous layer at the surface of cells has been viewed with skepticism; thus, no textbook of histology mentions it. However, the evidence presented here makes it clear that a "cell coat" exists at the surface of most, if not all, cells. Is it possible, then, to assign to the cell coat the properties which in the past have been vaguely attributed to the cell surface?

For instance, does the cell coat play a role in immunity? When antibodies prepared by injecting cells are given a fluorescent or ferritin label, they may be seen to combine with the surface of cells of the same type, where presumably the antigenic material is located (6, 45, 87). Now, this antigenic



Kidney. PA-silver stain.

FIGURE 37 *Brush border of proximal convoluted tubule.* The stained material present between the microvilli of the brush border (*bb*) may be followed within invaginations from the surface (*Inv*). The lower part of the picture is occupied by an intensely stained, unidentified "dense" body. $\times 38,000$.

FIGURE 38 *Proximal convoluted tubule.* This is an oblique section through the apical third of the cells. Two intercellular spaces filled with stained material appear as convoluted single lines indicated by arrows. Between these lines, the invaginations from the surface may be seen (*Inv*). $\times 46,500$.

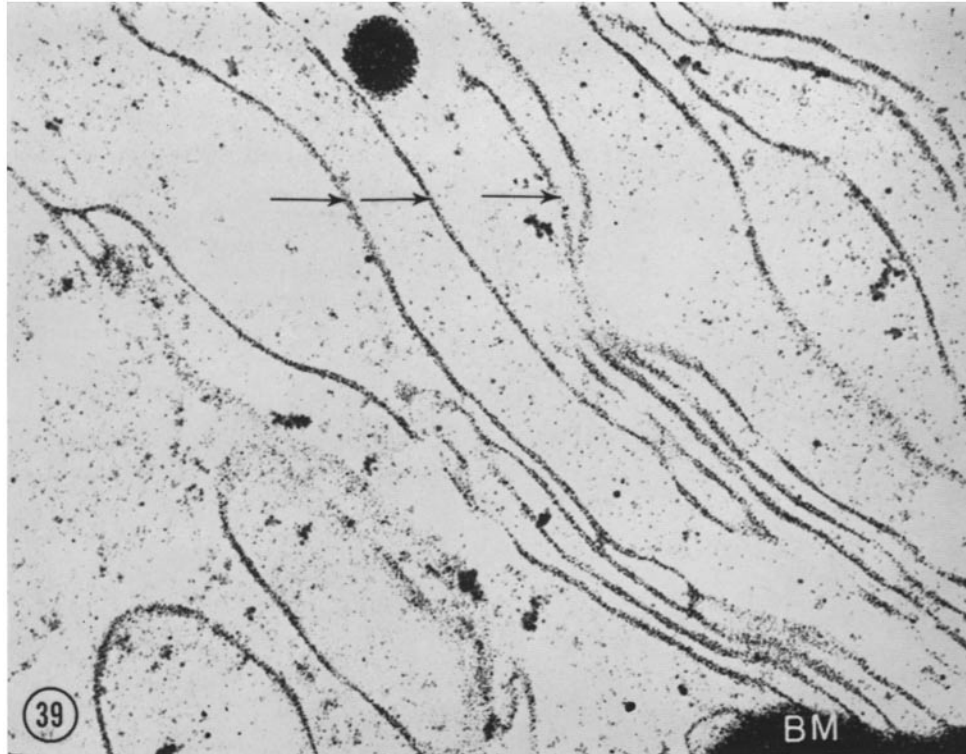


FIGURE 39 *Distal convoluted tubule: basal membrane infoldings.* From the strongly stained basement membrane (*BM*), the basal membrane infoldings may be followed as oblique dark lines (arrows) running to the upper left of the picture. The dark body at top may be a lysosome. $\times 40,000$.

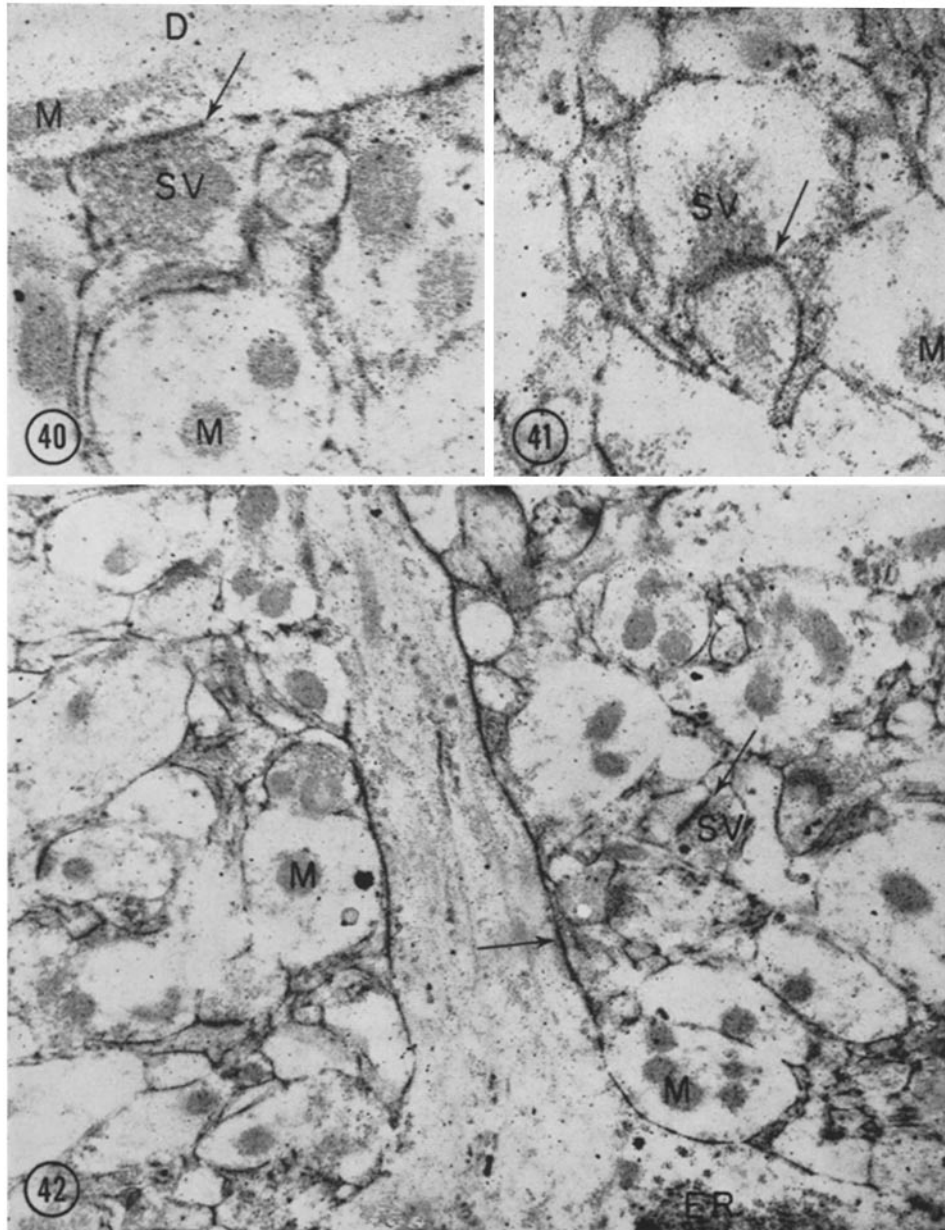
material is known to be associated with the enzyme ATPase (119, 120) which the electron microscope detects in exactly the same location as the cell coat (1, 44, 72, 89, 90, 115, 117).¹ The cell coat would thus seem to intervene in immunity.

¹ First, biochemical methods showed the cell membrane fraction to be rich in ATPase (29, 31, 108, 112, 121). Second, histochemical detection of ATPase in electron microscopy showed lead deposits outside the plasma membrane, filling the intercellular spaces of cerebral cortex (115), testis (113), liver (104), kidney tubules (117), colon (89, 90), retina (74), amphibian skin (36), toad urinary bladder (4), ureter (46), adrenal cortex (97), ciliary epithelium (59), and prostatic epithelium (70). In the striated border of intestinal epithelium and the brush border of the proximal convoluted tubule of kidney, ATPase was located between microvilli (1, 44, 117), whereas it occupied the extracellular space between the basal membrane infoldings of the distal convoluted tubule of kidney (1, 44, 72, 117). The similarity between the topography of this reaction and the results obtained with

Another possible function is suggested by the fact that in the *Drosophila* salivary gland the tight junction between cells is highly permeable to ions (and has low electrical resistance), whereas the rest of the cell surface has a low permeability to ions (and a high electrical resistance) (66). Since the tight junction is the only part of the surface which is not covered by cell coat, the high resistance of the rest may be due to *insulation* provided by this coat.

Finally, the proposal of Chambers that the surface material of cells is an *intercellular cement* (19, 20) received strong support from recent work in sponges. When these organisms were placed in water free from calcium and magnesium, the cells separated from one another; but when the dissociated cells were taken to a balanced medium to which a substance extracted from the original

PA-silver stain suggested that ATPase is part of the cell coat.



Cerebral cortex. PA-silver stain.

FIGURE 40 *Neuropile*. The staining of the intercellular space separating the dendrite (*D*) in the upper part of the picture from other processes is sharply increased in the region of the synaptic cleft (arrow). The matrix of mitochondria (*M*) and synaptic vesicles (*SV*) also take up some stain. $\times 42,000$.

FIGURE 41 *Synapse*. The synaptic cleft is indicated by an arrow. *SV*, synaptic vesicles. $\times 42,000$.

FIGURE 42 *Nerve cell*. The long dendrite of the nerve cell whose perikaryon is visible at the base is sharply outlined by cell coat material (arrow). In the neuropile, nerve and glial processes are separated from each other by stained material. A synaptic cleft is indicated by an oblique arrow. *M*, mitochondrion; *SV*, synaptic vesicles; *ER*, ergastoplasm. $\times 20,000$.

water had been added, they reaggregated (49, 50). In fact, it is now known that the substance is a glycoprotein normally present at the cell surface (75). This cementing glycoprotein may also be a component of the cell coat.²

² For over a century, it has been widely believed that an "intercellular cement" is responsible for the cohesion of cells. With the advent of the electron microscope, this belief lost popularity (18), as evidence was provided that cells are held together by desmosomes, terminal bars, and other similar attachments. The existence of a glycoprotein functioning as a cement between sponge cells (49, 50, 75) suggests that such a substance may also be present between rat cells. There were two possibilities: either the cell coats of adjacent cells would be glued together by such a substance, or the substance would be a component of the cell coats themselves, to which it would impart adhesive properties. In either case, the coats of the cells coming in contact would coalesce, and the stained interspace would appear as a single line (although the intercellular material should be loose enough to allow colloidal particles, and even mobile cells, to migrate; references 11, 15, 56-58, 71, 73, 93, 102). The stronger attachment observed at terminal bars and desmosomes might be due to an increased amount of the cementing substance, as suggested by a moderate increase in the thickness and density of the stained line in the wide portion of terminal bars (Fig. 37) and desmosomes (Fig. 24). Conversely, the poor staining at the tight junction of the terminal bar would suggest scarcity of cementing substance, the attachment in this location being due only to fusion of the outer leaflets of the plasma membranes.

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The difference in the staining of the coat of various cell types observed here as well as in previous work (98) suggests that the nature of the coats may differ from cell type to cell type. Indeed, Cox and Gessner showed that the mere addition of sugars to a culture medium may alter the cell surface and do so in a different manner in different cell lines (25). Furthermore, when several kinds of embryonic cells are mixed in tissue culture, the cells sort themselves out and build up structures reproducing the tissue of origin (83); the mutual identification implies that the cells of a given type have cell coats with similar surface properties. These properties might be altered. Thus, unlike normal lymphocytes, those lymphocytes whose surface sugars have been altered by treatment with glycosidase are removed from the circulation by reticulo-endothelial cells (42). Hence, the integrity of the cell coat would seem to be essential for the normal behavior of lymphocytes.

In summary then, the cell coat consists of material rich in glycoprotein and acidic residues, which possesses unique immunological, cementing, and other properties; and these may vary from cell type to cell type.

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