LANTHANUM STAINING OF THE SURFACE COAT OF CELLS

Its Enhancement by the Use of Fixatives Containing Alcian Blue or Cetylpyridinium Chloride

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ABSTRACT

Among the techniques which have been reported to stain the surface coat of cells, for electron microscopy, is lanthanum staining en bloc. Similarly, the presence of the cationic dye, Alcian blue 8GX, in a primary glutaraldehyde fixative has been reported to improve the preservation of the surface coat of cells of many types; however, the preserved coat is not very electron opaque unless thin sections are counterstained. The present paper shows that for several rat tissues lanthanum staining en bloc is an effective electron stain for the cell surface, giving excellent contrast, if combined sequentially with prefixation in an aldehyde fixative containing Alcian blue. The cationic substance cetylpyridinium chloride was found to have a similar effect to that of Alcian blue in enhancing the lanthanum staining of the surface coat material of the brush border of intestinal epithelial cells. The patterns of lanthanum staining obtained for the tissues studied strikingly resemble those reported in the literature where tissues are stained by several standard methods for demonstrating mucosubstances at the ultrastructural level. This fact and the reproduction of the effect of Alcian blue by cetylpyridinium chloride constitute a persuasive empirical argument that the material visualized is a mucopolysaccharide or mucopolysaccharide-protein complex.

INTRODUCTION

In 1963 Bennett proposed the comprehensive term "glycocalyx" to describe a polysaccharide-rich surface coating apparently present in many cells. The notion that the outside strata of the cell membrane might be associated with polysaccharide, in the case of the Schwann cell, had been adumbrated by Robertson in 1958. The cumulative cytochemical evidence for the presence of polysaccharide in the surface coat of cells, and for its ubiquity, has been reviewed in several recent papers (Rambourg and Leblond, 1967; Ito, 1969; Rambourg, 1969; Behnke and Zelander, 1970).

Among the techniques which have been reported to visualize the surface coat, e.g. in the intermediate line of nerve myelin, is that of lanthanum staining en bloc during fixation (Doggenweiler and Frenk, 1965; Revel and Karnovsky, 1966). However, Revel and Karnovsky (1967) found that the addition of alkaline lanthanum solutions to fixatives principally resulted in a nonspecific precipitation of lanthanum in the extracellular space, and found little evidence that lanthanum salts would bind to specific molecular components.

Recently Behnke (1968) and Behnke and Zelander (1970) have shown that the presence of the

cationic dye Alcian blue 8GX in the fixative solution greatly improves the preservation of the surface coat of cells. It is the purpose of the present paper to illustrate the effect of sequentially combining the use of fixatives containing Alcian blue, or the organic cationic substance cetylpyridinium chloride, with lanthanum staining en bloc, in delineating the surface coat of cells in a variety of tissues. A preliminary report of the combination of fixatives containing Alcian blue with lanthanum staining en bloc has already appeared (Shea and Karnovsky, 1969).

MATERIALS AND METHODS

Young male rats (150–250 g) of the Sprague-Dawley (Holtzman) strain were used in all experiments. Primary fixation of tissues was either by immersion of blocks of tissue, or by vascular perfusion. Before operation or sacrifice, all rats were anesthetized by the intraperitoneal injection of 0.2 ml/100 g body weight of a 5% solution of sodium pentobarbital ("Nembutal sodium", Abbott Laboratories, Chemical Marketing Div., North Chicago, Ill.).

Perfusion Fixation

The apparatus consisted of two 500 ml reservoirs (one containing fixative and the other containing physiological saline) connected to a short length of vinyl tubing by "Plexitron" intravenous solution administration sets (Travenol Laboratories, Inc., Deerfield, Ill.) and a three-way stopcock (Becton-Dickinson & Company, Rutherford, N. J., BD-MS02). The vinyl tubing in turn was connected to a polyethylene cannula ("Intramedic" polyethylene tubing, PE 60 or PE 100, Clay-Adams, Inc., Parsippany, N. J.) by a plastic tubing adaptor (Clay-Adams, Inc.). With this apparatus it was possible to ensure, without using the stopcock during the perfusion, that the flow of fixative into the perfused organ was preceded by that of 2–3 ml of physiological saline.

The fixative was a 3% solution of glutaraldehyde in a modified (half-strength) physiological saline solution (cf. Maunsbach, 1966), containing 0.02 M cacodylate buffer at pH 7.2, and was kept at room temperature. The final osmolality was 560 mosmoles. In the case of kidney perfusions this fixative contained 0.5% Alcian blue 8GX (Allied Chemical Corp., Industrial Chemicals Div., Morristown, N. J.).

For perfusion of the liver, after surgical exposure of the liver, the gut was displaced to reveal the superior mesenteric and portal veins. A loose silk ligature was placed so as to encircle the portal vein, and *also* to encircle the hepatic artery. A polyethylene cannula (PE 100) was inserted into the portal vein through an opening made in the superior mesenteric vein, and secured by tightening the ligature, which also occluded the hepatic artery. The inferior vena cava was incised, and the liver was perfused at a pressure of 8 mm Hg.

For perfusion of the left kidney, a silk ligature was placed around the abdominal aorta below the left renal artery, and another immediately above the left renal artery and below the right renal artery and other principal abdominal arteries. A polyethylene cannula (PE 60) was inserted through an incision in the lower abdominal aorta, and secured by tightening the lower ligature. The upper ligature was immediately tied. The inferior vena cava was incised. The perfusion pressure was 120–150 mm Hg.

5–10 min after the start of the perfusion, 1 mm blocks of liver or kidney were minced and fixed in the same glutaraldehyde fixative, containing 0.5% Alcian blue, for 2 hr at room temperature. Postfixation in osmium tetroxide–lanthanum was preceded by a brief rinse in 0.1 M cacodylate buffer at pH 7.2.

Immersion Fixation

For immersion fixation, blocks of tissue were fixed either in a formaldehyde-glutaraldehyde fixative (Karnovsky, 1965) diluted (1:1) with 0.1 M cacodylate buffer, at pH 7.2 (Graham and Karnovsky, 1966), or in a 3% glutaraldehyde solution in 0.1 M cacodylate buffer, at pH 7.2. Alcian blue (0.1 or 0.5%), or cetylpyridinium chloride (Pfaltz and Bauer, Inc., Corona, N. Y.) (0.5%), was added as indicated in the legends. Fixation was carried out for 2 hr at room temperature. Postfixation in osmium tetroxide–lanthanum was preceded by a brief rinse in 0.1 M cacodylate buffer at pH 7.2.

Postfixation and Lanthanum Staining

Postfixation with lanthanum staining en bloc (Revel and Karnovsky, 1967) was in a solution containing 1% osmium tetroxide and 1% lanthanum nitrate (the hexahydrate, Fisher Scientific Company, Pittsburgh, Pa.), in 0.1 M *s*-collidine buffered at pH 8.0. The final pH of the solution of osmium tetroxide and lanthanum nitrate in collidine buffer was pH 8.05. Fixation was carried out for 2 hr at room temperature. Tissue was dehydrated in ethanol and propylene oxide, and embedded in Epon 812. Thin sections were cut with glass knives on a Sorvall MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.), and examined in an RCA EMU-3F electron microscope. Except for the section illustrated in Fig. 1, which was lightly stained with lead, the sections were studied unstained.

OBSERVATIONS

Endothelial Cells

Immersion fixation of blocks of skeletal muscle in an aldehyde fixative containing Alcian blue,

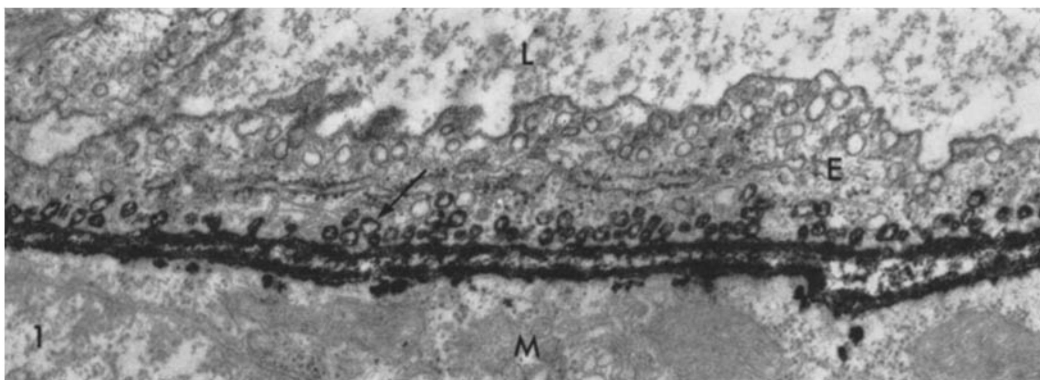


FIGURE 1 Rat cremaster muscle, capillary. Fixation was in formaldehyde-glutaraldehyde with added Alcian blue (0.1%), with postfixation in OsO_4 combined with lanthanum staining en bloc. The section was lightly stained with lead. A lanthanum precipitate permeates the extracellular space and basement membranes separating the endothelial cell (*E*) and muscle fiber (*M*). Many endothelial vesicles near the basement membrane contain lanthanum, including some vesicles obviously opening on to the cell surface, and others apparently free in the cytoplasm; the latter probably connect with the plasmalemma in a different plane of section, or through a vesicular chain. In many vesicles (arrow) the lanthanum staining appears to be confined to a "surface coat" lining the vesicle. *L*, lumen. × 28,000.

followed by lanthanum staining en bloc, led to lanthanum staining of superficial capillaries, which was sometimes confined to the abluminal capillary front. Lanthanum, as well as forming a precipitate in the basement membrane, stained a surface coat, perhaps best recognized in those plasmalemmal vesicles presumably communicating with the cell surface but not completely filled with a lanthanum precipitate (Fig. 1). In this figure, the arrow points to such a stained vesicle, while immediately to the right of the arrow is an unstained vesicle which is presumably free in the cytoplasm.

Liver Sinusoids

Perfusion fixation of liver with a glutaraldehyde fixative maintained an open sinusoidal structure, and permitted a deeper penetration of Alcian blue and of lanthanum into the blocks during the later stages of fixation than was obtainable by immersion fixation alone. The surfaces of the microvilli of hepatocytes, and the surfaces of endothelial cells, were stained with lanthanum, as well as those of the fibers of occasional collagen bundles (Fig. 2). There was not always a homogeneous precipitate of lanthanum filling the space of Disse.

Kidney

Primary fixation was either by immersion of blocks in an aldehyde fixative solution containing Alcian blue (for the study of the surface coat of tubular cells), or by the perfusion of a glutaraldehyde solution containing Alcian blue (for the study of glomeruli). As in both cases tissues were exposed to lanthanum en bloc during postfixation with osmium tetroxide, the best lanthanum staining was obtained in sections from the superficial layers of the tissue blocks.

In proximal tubular cells there was intense lanthanum staining of the surfaces of the microvilli of the brush border (Fig. 3). In glomeruli there was staining of the surfaces of the endothelial cells and of the podocytes, and less consistently a heavy precipitate of lanthanum in the basement membrane (Fig. 4). The dense staining of the surfaces of the foot processes extends into the slit pores, in which the stained surface layers often appear quite closely apposed (Fig. 4).

Small Intestine

Lanthanum staining of blocks of small intestine, without prior fixation in fixatives containing Alcian blue or cetylpyridinium chloride, led occasionally to staining of the surface coat of the microvilli of the brush borders of the absorptive cells, but this staining was poorly reproducible and not intense; a well-stained example is shown in Fig. 7. If, however, the blocks were fixed in glutaraldehyde containing Alcian blue (Fig. 5), or cetylpyridinium chloride (Fig. 6), before lanthanum staining en bloc, the surface coat of the microvilli was consistently and intensely stained.

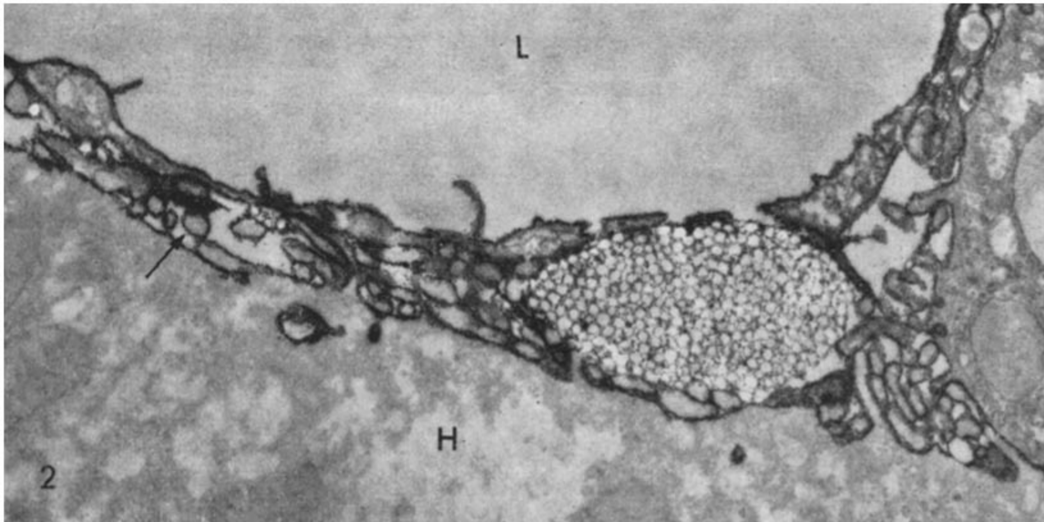


FIGURE 2 Sinusoidal region of rat liver fixed by perfusion with a glutaraldehyde solution, quickly followed by immersion fixation in a glutaraldehyde solution containing Alcian blue (0.5%), and postfixed with OsO_4 with lanthanum staining en bloc. The section was unstained. Note the opacity of the endothelial cell surface membranes, and of the surface membranes (arrow) of the microvilli of the hepatocyte (*H*). *L*, lumen of the sinusoid. × 17,000.

The apical surface coat extends an appreciable distance beyond the tips of the microvilli and stains well with lanthanum, if lanthanum staining en bloc is preceded by fixation in glutaraldehyde containing Alcian blue, or cetylpyridinium chloride (Fig. 8).

DISCUSSION

The partial preservation of mucopolysaccharides in tissues fixed with formaldehyde has been attributed to a reaction of formaldehyde, not with mucopolysaccharides, but with structurally associated proteins (Szirmai, 1963). Behnke and Zelander (1970) remark that the affinity of aldehyde and osmium tetroxide fixatives for mucopolysaccharides is poor, and ascribe the difficulty in demonstrating surface coat substance in the cells of conventionally fixed mammalian tissues to faulty fixation. Behnke and Zelander (1970) have shown, however, that the addition of Alcian blue to a glutaraldehyde fixative greatly improves the preservation of a material present in the surface coat of cells of many types; this material they call a "mucosubstance", and regard as a polysaccharide-protein complex. In using Alcian blue to preserve the surface coat material, Behnke and Zelander (1970) believe that they are exploiting the property of cationic substances of precipitating mucopolysaccharides, as recommended by Szirmai (1963). Analogously, Williams and Jackson (1956), who made use of Scott's (1955) observation that acid mucopolysaccharides are precipitated by cetylpyridinium chloride, reported improved preservation of PAS[1]-reacting tissue components for light microscopy, when this cationic substance was added to a formaldehyde fixative.

The observations presented above show that lanthanum is an effective electron-opaque stain for surface coat material in several tissues, if primary fixation is in glutaraldehyde or formaldehyde-glutaraldehyde fixatives containing Alcian blue. Cetylpyridinium chloride was found to have an effect similar to that of Alcian blue in enhancing the lanthanum staining of the surface coat material of the brush border of intestinal epithelial cells.

The precise role of lanthanum in staining of the cell surface is uncertain. Doggenweiler and Frenk (1965) assumed it to stain lipid, and Lesseps (1967) considered the lanthanum-staining material to contain lipid, and possibility to contain polysaccharides and protein as well. Behnke (1968) has assumed lanthanum staining to be specific

[1] *Abbreviations:* PAS, periodic acid–Schiff (test).

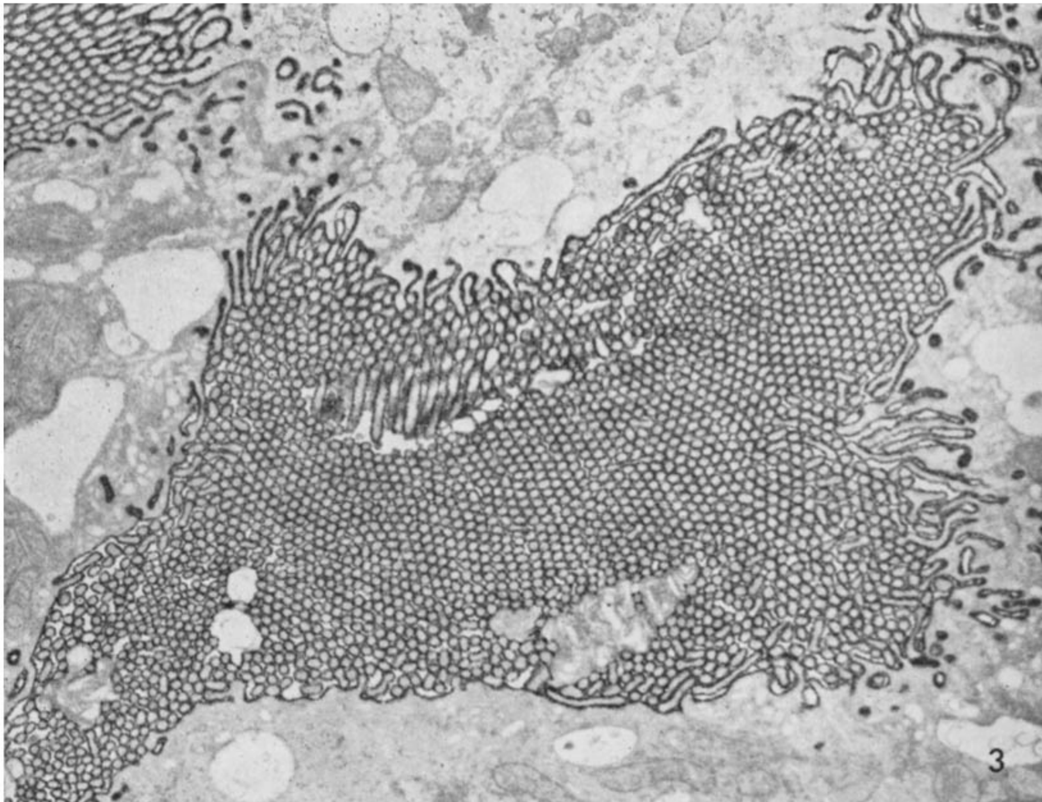


FIGURE 3 Brush border of cell of proximal convoluted tubule, rat kidney. Fixation was in formaldehyde-glutaraldehyde with Alcian blue (0.1%), with postfixation in OsO_4 combined with lanthanum staining en bloc. The section was unstained. Note the opacity of the surface membranes of the microvilli. × 12,000.

or the demonstration of acidic mucosubstances at the ultrastructural level. Its enhancement by fixatives containing Alcian blue or cetylpyridinium chloride, as described above, argues in this sense; this point will be further discussed below.

As Behnke and Zelander (1970) point out, any specificity of the effect of Alcian blue used in this way is related to its effect as a precipitant of mucosubstances, and is distinct from the specific staining of histological materials by Alcian blue at low pH.

Alcian blue has in itself possibilities as an electron stain. Tice and Barrnett (1965), who used Alcian blue at pH 3.0 to stain thin sections, showed increased density of the material between the microvilli of intestinal absorptive cells. Similarly, Rothman (1969) showed staining of the epicuticle of the worm *Moniliformis dubius*; tissue had been fixed in a formaldehyde fixative, and stained en bloc with Alcian blue at pH 2.5. Behnke and Zelander (1970), however, found that the presence of Alcian blue in a glutaraldehyde fixative conferred little opacity to electrons on the surface coat material of rat cells, even with postfixation in osmium tetroxide; it was necessary to stain thin sections with uranyl acetate and lead citrate to provide contrast. This agrees with our experience with control rat tissues fixed in glutaraldehyde–Alcian blue, and postfixed with osmium tetroxide *without* lanthanum staining en bloc.

These results perhaps also account for the varieties of lanthanum staining or labeling which have been reported in the literature: for the staining of surface membranes described by several authors (Doggenweiler et al., 1964; Doggenweiler and Frenk, 1965; and Revel and Karnovsky, 1966), as well as for delineation of the extracellular space by a nonspecific precipitate of lanthanum (Revel

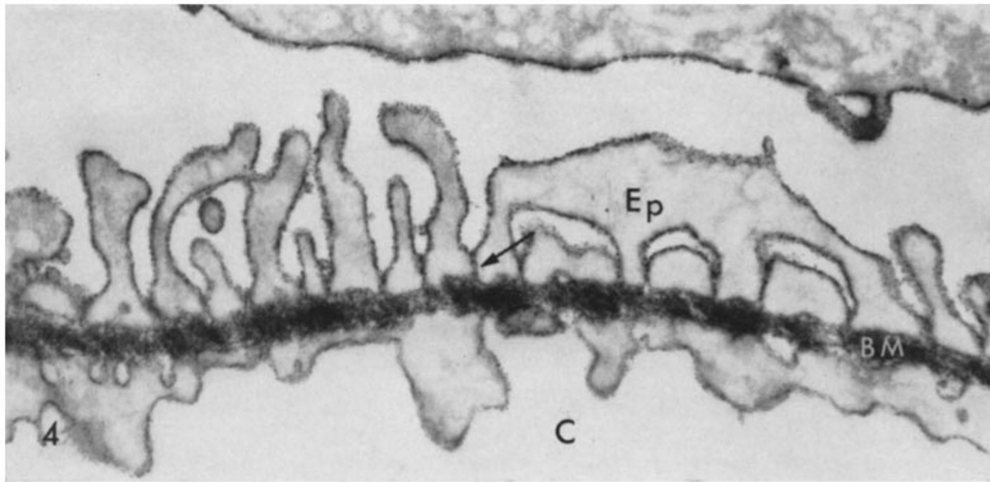
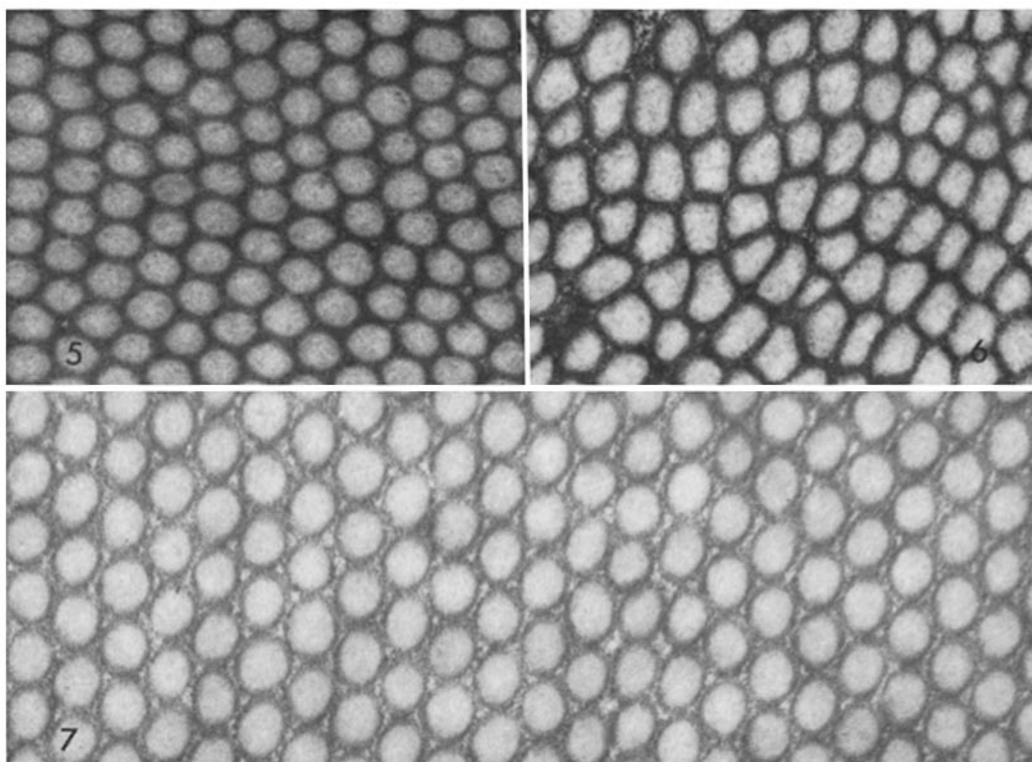


FIGURE 4 Peripheral capillary wall of renal glomerulus, rat. The kidney was fixed by perfusion with a glutaraldehyde solution containing Alcian blue (0.5%); postfixation was in OsO_4, and was combined with lanthanum staining en bloc. The section was unstained. The urinary space is above; it contains epithelial cells (*Ep*), and their foot processes which are separated by slit pores (arrow) and rest on the basement membrane (*BM*). The latter is separated by the endothelium from the capillary lumen (*C*). Note the heavy precipitate of lanthanum in the basement membrane, and the increased opacity of the surface membranes of the endothelium, and of the epithelial cells and of their foot processes. Note also that in some of these images of slit pores (arrow) the stained surface membranes of adjacent foot processes appear to be closely apposed. × 26,000.

and Karnovsky, 1967). The former staining effect, we believe, is represented in the staining of the surface membranes of cells in Figs. 1–8, while the latter "tracer" effect is perhaps represented by the presence of lanthanum in the basement membrane (Figs. 1 and 4) (see below). The staining of the cell surfaces with lanthanum we ascribe to the presence in the cell surface coat of acid mucopolysaccharides, perhaps in the form of mucosubstances or proteoglycans (Spiro, 1970; Rodén, 1968), and its enhancement by Alcian blue and by cetylpyridinium chloride we ascribe to the known precipitation of acid mucopolysaccharides by cationic substances (Szirmai, 1963).

Gasic and Gasic (1962, 1963) and Gasic and Berwick (1963) have provided cytochemical evidence for the presence of sialic acid in the cell coat of ascitic tumor cells. In the case of the surface coat of the brush border of intestinal absorptive cells, cytochemical evidence has been adduced for the presence of both sulphate (Ito, 1969) and carboxyl (Ito, 1965) polyanions in the mucopolysaccharide of the surface coat, though Scott and Dorling (1965) report that the polyanions of ileum are characteristically carboxylates. In either case, a protein polysaccharide component could account for lanthanum staining, just as lanthanum is known to precipitate certain protein polysaccharides (Doganges and Schubert, 1964). Against this interpretation of the chemical basis of lanthanum staining of the cell surface coat can be set the fact that cetylpyridinium chloride is a potent blocking agent for the staining of the acidic mucopolysaccharide of cartilage by Alcian blue (Quintarelli et al., 1964). If lanthanum staining of the cell surface depends, like Alcian blue staining of polyanions, on the formation of "salt links" (Scott et al., 1964), then staining of the cell surface with lanthanum after exposure of tissues to fixatives containing cetylpyridinium chloride might well seem paradoxical. The paradox would be removed if lanthanum ions in the concentration employed (0.023 M) were to serve as an "unblocking" agent for at least some of the protein polysaccharides of the cell surface, just as potassium (Kelly et al., 1963) and magnesium salts (Quintarelli et al., 1964) have been reported to unblock the staining of cartilage, with toluidine blue and with Alcian blue, respectively, after it has been blocked with cetylpyridinium chloride.



FIGURES 5–7 Tangential sections of microvilli of brush border of intestinal absorptive cells, rat ileum. Postfixation was in OsO_4, and combined with lanthanum staining. The sections were unstained. Lanthanum staining, en bloc, was preceded by fixation in glutaraldehyde with added Alcian blue (0.5%) (Fig. 5), or cetylpyridinium chloride (0.5%) (Fig. 6), or in glutaraldehyde without either additive (Fig. 7). Note that the interstitial material between the microvilli is much increased in opacity when Alcian blue or cetylpyridinium chloride is used in conjunction with lanthanum (Figs. 5–6), in contrast with the less intense and less uniform staining with lanthanum alone (Fig. 7). Figs. 5–7, × 53,000.

Staining of basement membranes by lanthanum after fixation with glutaraldehyde–Alcian blue may have a different basis from staining of the cell surface. There was little evidence, in comparing results obtained with glomerular basement membrane with staining of the cell surface coats, e.g. of podocytes, of comparably predictable staining of the basement membrane. The results rather resembled those obtained in labeling the extracellular space, by the use of alkaline lanthanum solutions, by Revel and Karnovsky (1967), since the lanthanum seemed easily leached out of the glomerular basement membrane. This would be consistent with the relatively low concentration of sialic acid (1.20% dry weight) in glomerular basement membrane (Spiro, 1967 *a*, *b*; Mohos and Skoza, 1969). Recently Mohos and Skoza (1970) have suggested that even this figure may be due in part to contamination of basement membrane preparations by cell membranes at the centrifugal forces used to collect glomeruli, and suggest that the sialic acid content of glomerular basement membrane (0.27–0.68% dry weight) obtained by Misra and Berman (1966), using low centrifugal forces, may be more accurate. Mohos and Skoza (1970) suggest that the basement membrane proper may not contain sialic acid in sufficient quantity for histochemical demonstration.

The staining of the surface of endothelial cells by lanthanum after fixation in fixatives containing Alcian blue (Figs. 1, 2, and 4) recalls the result reported by Luft (1965) with ruthenium red (Luft, 1964). Analogous staining of the surface of endothelium has been obtained by Wetzel et al.

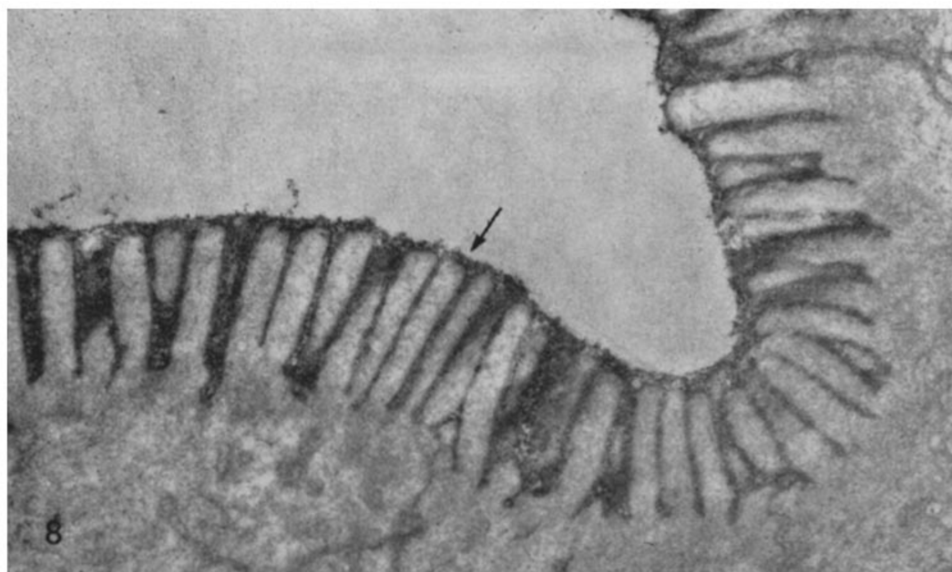


FIGURE 8 Brush border of intestinal absorptive cells, rat ileum. Fixation as in Fig. 6. The section was unstained. Note the increased opacity of the interstitial material present between the microvilli and extending as a surface coat (arrow) beyond the tips of the microvilli. × 36,000.

(1966) with dialyzed iron, by Rambourg and Leblond (1967) with colloidal iron, and by Rambourg (1969) with a chromic–phosphotungstic acid stain. In tissues fixed with glutaraldehyde–Alcian blue, with postfixation in osmium tetroxide, a dense surface layer is also observed, provided the sections are stained with uranyl and lead (Behnke and Zelander, 1970).

The staining of the cell surface of the podocytes of the glomerulus (Fig. 4) corresponds in its pattern to results obtained with the colloidal thorium method of Revel (1964) and with silver methenamine (Rambourg and Leblond, 1967); with colloidal iron (Jones, 1969; Groniowski et al., 1969); with ruthenium red (Groniowski et al., 1969); and with glutaraldehyde–Alcian blue fixation (Behnke and Zelander, 1970) where contrast was obtained by postosmication and staining of thin sections with uranyl and lead. However, the pattern of approximation of the surface coats of adjacent foot processes at the slit pores, so far as can be determined at relatively low magnification (Fig. 4), corresponds rather well to the diagram of the relations between the foot processes in vivo postulated by Latta (1970) (in his Fig. 12).

The pattern of staining of the surface coat of the microvilli of renal tubular cells (Fig. 3) resembles that obtained by Groniowski et al. (1969) with colloidal iron, ruthenium red, and colloidal thorium, and by Rambourg and Leblond (1967) with silver methenamine. Similarly, the pattern of staining of the microvilli of intestinal absorptive cells (Figs. 5–8) resembles that obtained with colloidal (Curran et al., 1965) and dialyzed (Wetzel et al., 1966) iron, with colloidal thorium (Ito, 1965), silver methenamine (Rambourg and Leblond, 1967), ruthenium red (Luft, 1964; Behnke and Zelander, 1970), and Alcian blue (Tice and Barrnett, 1965); and with fixation with glutaraldehyde–Alcian blue followed by staining with uranyl and lead (Behnke and Zelander, 1970). In the case of renal tubular and intestinal absorptive cells the surface coat of the brush border has also been stained with phosphotungstic acid (Pease, 1966), though the precise chemical interpretation of this effect is in dispute (Glick and Scott, 1970; Pease, 1970; Scott and Glick, 1971).

When lanthanum staining en bloc of intestinal absorptive cells is not preceded by exposure of the blocks to Alcian blue or to cetylpyridinium chloride (Fig. 7), as remarked above, it does not give rise to intense or reproducible staining of the surface coat of the microvilli. We have been unable to confirm the patchy pattern of lanthanum staining obtained by Overton (1968) in the duodenal brush border of the mouse. Possibly this difference

is attributable to technical differences, such as in the pH of the lanthanum solutions used; Overton (1968) used a glutaraldehyde fixative containing lanthanum, at pH 7.2. However, when lanthanum staining en bloc of intestinal absorptive cells is preceded by fixation in fixatives containing Alcian blue or cetylpyridinium chloride (Figs. 5, 6, and 8), the density of the surface coat material compares very favorably with that obtained with ruthenium red (Behnke and Zelander, 1970), and that obtained with the immunologically specific method of Bernhard and Avrameas (1971).

Behnke and Zelander (1970) review the techniques that have been used in recent years to identify mucosubstances at the ultrastructural level, and tabulate the results obtained with a wide variety of methods and tissues. They conclude that the use of Alcian blue in aldehyde fixatives leads to the preservation of electron-opaque material in sites agreeing with those identified by many cytochemical methods for mucosubstances. By combining this method of fixation with lanthanum staining en bloc, we have obtained a comparable pattern of staining of high sensitivity. This effect of Alcian blue and its reproduction by cetylpyridinium chloride constitute a persuasive empirical argument that the material visualized is a mucopolysaccharide or mucopolysaccharide-protein complex. The precise chemical basis of the lanthanum staining remains to be established.

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