ELECTRON MICROSCOPE STUDIES ON
THE SURFACE COAT OF THE NEPHRON

J. GRONIOWSKI, W. BICZYSKOWA, and M. WALSKI
From the Department of Pathology, Medical Academy, Warsaw, Poland

ABSTRACT

Attempts to make visible the carbohydrate coat at the free cell surface of glomeruli as well as the tubules of rabbit kidney were undertaken. The ruthenium red procedure was performed, according to Luft, at various pH values. Moreover, the colloidal iron and the colloidal thorium methods were used. Neuraminidase digestion was also performed. In the ruthenium red procedure the luminal face of the epithelial cells of the nephron was coated distinctly with reaction product. The results obtained revealed that some of the differences at various levels of the nephron depended on the pH values. In glomeruli and proximal convoluted tubules the optimum pH value was 7.4; in the ascending limb of Henle loops and distal convoluted tubules the optimum pH value was 6.8. The ruthenium red-positive surface coat was either closely connected with, or appeared as a part of, the outer leaflet of the unit membrane. The slit pores of glomeruli were also covered by a coat continuous with the surface coat of the adjacent foot processes. The coat lining the microvilli of proximal convoluted tubules completely filled the intervillous spaces. Also, both the colloidal iron method and the colloidal thorium method evidenced the presence of surface coat. Pretreatment with neuraminidase abolished the effect of the Hale reaction. These results may indicate that the surface coat of the epithelia of the nephron shows the presence of glycoproteins containing sialic acid residues.

In the last few years, with the use of the electron microscope technique the presence of a surface coat rich in complex carbohydrates has been described for various epithelial and nonepithelial cells (2, 9, 11, 12, 17, 18, 20, 25, 26). Attempts have been made to make visible the carbohydrate-rich coat at the cell surface of the glomeruli and tubuli as well. The presence of an extracellular substance between the microvilli of renal tubular cells was demonstrated on routine electron micrographs (6, 21), and the glycoprotein nature of that substance has been postulated. However, the extraneous coats of various cell surfaces could not be identified at the ultrastructural level until some methods were introduced for making the glycoproteins visible. Using the “inert dehydration” procedure with glycol and staining with phosphotungstic acid, Pease (22) succeeded in demonstrating the surface coat of the epithelial cells in the rat kidney. Since Rambourg found evidence that phosphotungstic acid is nearly specific for glycoproteins (Rambourg, A. Personal communication.), a component of that nature in the surface coat could be accepted. Rambourg and Leblond (23), by means of the colloidal thorium procedure, revealed the existence of the surface coat; the surfaces of podocyte foot processes, as well as the free surface of the capillary endothelium, were heavily coated with thorium par-
ticles. The same authors showed the presence of a delicate extraneous coat at the free surfaces of the proximal convoluted tubuli by the use of the periodic acid-silver methenamine technique. Also, the colloidal iron technique adapted to electron microscopy enabled demonstration of glycopro-

**Figure 1 a** Continuous coat at the surface of a podocyte. The surface coat at the foot processes seems to be most prominent (arrows). Ruthenium red procedure. X 64,000.
The purpose of this report has been to extend in some way the observations of the extraneous coats of epithelial cells and, in particular, to compare these coats at various levels of the nephron. The ruthenium red procedure introduced by Luft (16) can be used for demonstration of a variety of extracellular materials (13, 17, 18). Since the extraneous coat stained with ruthenium red could be easily traced, that method was used in this study. In addition, the colloidal iron method and colloidal thorium technique were used.

**Figure 1b** At higher magnification the ruthenium red-marked material of the surface coat seems to arise from, or to be closely attached to, the outer leaflet of the unit membrane. The slit pores are covered by the material continuous with the surface coat of the adjacent foot processes (arrows). $\times 154,000$. 

J. Goniowski, W. Biczyskowa, and M. Walski Nephron Surface Coat 587
Figure 2  Electron-lucent interzone between the ruthenium red-marked outer leaflet and the inner leaflet is conspicuous. × 175,000.
Figure 3  Epithelium of the proximal convoluted tubule in ruthenium red procedure. Brush border, b; nucleus, n; intercellular space, s; basement membrane, bm; capillary lumen, cl. X 6,000.
FIGURE 4 Detail of the epithelial cells of the proximal convoluted tubule. The cell membrane of microvilli is marked by the reaction product. The granular deposit of stained material is present in intercellular spaces. Ruthenium red procedure. X 63,000.

MATERIALS AND METHODS

The investigations were carried out on nine adult rabbits. Small fragments from the cortical part of the kidney were obtained from the animals under deep Nembutal anesthesia. The following methods of study were used: ruthenium red procedure (19), colloidal iron method (9), colloidal thorium method (23), and, in addition, digestion with neuraminidase and a standard morphological examination were performed.

Staining of tissue blocks with ruthenium red was carried out according to the method of Luft (19). However, the method was modified by the use of three different pH values for the first as well as for the second group of fixing solutions. In each experiment different tissue samples were fixed for 1 hr at 4°C in three fixing solutions, respectively: 1.2% glutaraldehyde containing 3000 p.p.m. ruthenium red buffered with cacodylate at pH 6.8; 1.2% glutaraldehyde containing 3000 p.p.m. ruthenium red buffered with cacodylate at pH 7.0; and 1.2% glutaraldehyde containing 3000 p.p.m. ruthenium red buffered with cacodylate at pH 7.4.

After the tissue had been rinsed three times, over a period of 10 min, with a change of the appropriate cacodylate buffer each time, it was then fixed again for 3 hr at room temperature in an osmium tetroxide-ruthenium red solution in the appropriate cacodylate buffer.

Three different fixing solutions were used, respectively:

1 Twice before the procedure, the kidney was perfused briefly through the renal artery with the mixture at pH 7.4.
tively: 1.7% osmium tetroxide containing 3000 p.p.m.
ruthenium red buffered with cacodylate at pH 6.8;
1.7% osmium tetroxide containing 300 p.p.m. ru-
thenium red buffered with cacodylate at pH 7.0; and
1.7% osmium tetroxide containing 3000 p.p.m. ru-
thenium red buffered with cacodylate at pH 7.4.

The material was rinsed briefly in the appropriate
buffer, dehydrated with ethanol, and then embedded
either in Epon or in Vestopal W. Sections were

treated with lead hydroxide.

The colloidal iron method, as a modification of the
method of Gasic and Berwick (9), was performed on
small tissue samples fixed for 2 hr in a 3.6% glutar-
aldehyde solution buffered with cacodylate at pH 7.3.
The samples were incubated in colloidal iron solution
for 1 hr at pH 1.5, after which they were dehydrated
and embedded either in Vestopal W or in Epon.

The colloidal thorium method, according to
Rambourg and Leblond (23), was carried out on
small tissue samples fixed for 2 hr at 4°C in 2.5%
glutaraldehyde solution in phosphate buffer at pH
6.9. Next, the samples were washed and postfixed in
2% OsO₄ in phosphate buffer at pH 6.9 and subse-
duently incubated for 24 hr at room temperature in
1% Thorotrast solution in 3% acetic acid at pH 2.6.
After dehydration the samples were embedded in
Epon.

Neuraminidase digestions (Neuraminidase; Beh-
ringwerke, Marburg, West Germany) were per-
formed under two different conditions. Small tissue
samples were incubated for 24 hr at 37°C in the fol-

gowing mixtures respectively: 0.1 M phosphate buffer
at pH 7.0 containing 250 u neuraminidase/ml, and
0.1 M phosphate buffer at pH 6.5 containing 250 u
neuraminidase/ml. Samples were fixed in 2.5%
glutaraldehyde solution, and the reactions with col-

Figure 5 Cross-section of brush
border. Microvilli are lined by a dis-
tinct coat which fills the intervilous
spaces. Ruthenium red procedure.
× 96,000.
FIGURE 6  Microvilli of proximal convoluted tubule are lined with a delicate coat. This coat may be followed within invaginations from the surface (arrows). Intercellular spaces are heavily filled with the stained material. Ruthenium red procedure. X 35,000.
loidal thorium and colloidal iron were performed. Then they were dehydrated and embedded in Vestopal W or Epon. Tissues incubated in buffers without neuraminidase at two different pH values were used as controls.

Samples for the routine procedure were fixed in 3.6% glutaraldehyde solution buffered with cacodylate at pH 7.3. Next, they were washed and postfixed in 1% osmium tetroxide in cacodylate buffer of the same pH, and then dehydrated and embedded in Vestopal W or in Epon.

All the blocks were cut with the Porter-Blum or Reichert OMU 2 ultramicrotome, and sections were examined with the JEM 7 electron microscope.

RESULTS

With the ruthenium red procedure, the luminal face of the epithelial cells was coated with a more or less distinct surface coat. The procedure revealed that some differences in the results obtained at various levels of the nephron depended upon the pH values. In glomeruli and proximal segments of the tubular nephron the most consistent and abundant deposition of the reaction product was obtained in the tissue that had been treated at pH 7.4 (see Fig. 1 a). In the ascending limb of Henle loops and in distal convoluted tubuli, the optimum pH value was 6.8 (Fig. 7).

The glomeruli were regularly a site of the reaction product. The surface coat observed regularly on the epithelial cell foot processes seemed to be distinctly marked (Fig. 1 a). The ruthenium red-positive surface coat was closely connected with, and appeared as a part of, the outer leaflet of the "unit membrane" (the cell membrane) and was of the same density as the outer leaflet (Figs. 1 b and 2). The average thickness of the surface coat of the foot processes was 120 A, including the outer leaflet of the cell membrane. The slit pores were covered by a coat continuous with the surface coat of the adjacent foot processes. The site of attachment of the foot processes to the outer part of the basement membrane revealed a small amount of the reaction product scattered at random within the basement membrane. Slight deposits were seen also on the endothelial lining of glomerular capillaries.

In the tubular part of the nephron the intensity of the surface coat was closely related to the localization and amount of microvilli. A distinct surface coat was observed on the luminal face of proximal convoluted tubules (Fig. 4). In cross-sections the surface coat appeared to be located on the outer leaflet of microvilli (Fig. 5). The sur-

![Figure 7](image-url) Epithelium of the distal convoluted tubule. Ruthenium red procedure. Luminal face of the cells is coated with the stained material. × 50,000.

J. Groniowski, W. Biczyskowa, and M. Walski Nephron Surface Coat
face coat covered all the intervillous spaces. The stained material that was present between the microvilli of the brush border of the proximal convoluted tubules could be followed within the invaginations of the cell surface which occurred frequently at the base of the microvilli. The invaginations run a sinuous course and appear as discontinuous tubular or vesicular profiles in the apical region of the cell (Fig. 6).

The reaction product was localized between

---

**Figure 8** A detail of the epithelial cell of the proximal convoluted tubule. Granular deposit of the stained material is present in basal infoldings of the cell (arrows). Ruthenium red procedure. × 50,000.
the adjacent epithelial cells of the tubules. It usually had a higher density than the surface coat of the free cell surfaces (Figs. 3, 4, 6). The intercellular spaces were either delineated at the cell membrane by the reaction product or completely filled with the deposits. The reaction product was also present at the terminal bars, yet it was absent at the level of "tight junctions."

The other site of the reaction product in the proximal and distal convoluted tubules was at the base of the cells. The deposits appeared regularly at the basal infoldings of the cell membrane (Fig. 8). Since the infoldings were more numerous and extended deeper in the cells of the ascending limbs of Henle loops and of the distal convoluted tubules than in the cells of the proximal convoluted tubules, the deposits were much more distinctly marked in the former cells. As a rule, irregular granular deposits were observed in the adjacent basement membrane (Fig. 9). When the endothelial lining of capillaries was damaged, the basement membrane was heavily stained (Fig. 10).

Sites of the reaction product were not observed on structures within the epithelial cells. Slight deposits were seen on the endothelial lining of the capillaries. They were relatively more abundant on the luminal face of the endothelial cell membranes of arterioles. In sections from kidneys prefixed by perfusion a large amount of dense reaction product was present in micropinocytotic vesicles of endothelial cells (Fig. 11) These vesicles were localized near the luminal surface.

With the colloidal iron procedure the Hale-positive material gave good evidence for the existence of a surface coat on the epithelial cells of the nephron. In glomeruli, the material formed a continuous coat at the surfaces of podocytes (Fig. 12). Also the slit pores were covered by a delicate lining of iron deposit. In the tubular part of the nephron the luminal face of epithelium was covered by an iron-positive layer. Also the

![Figure 9](image9.png)
**Figure 9** Deposit of the stained material in basal infoldings. Granular deposit of the stained material in the adjacent part of the basal membrane (arrows). Endothelial cell, e; capillary lumen, cl. Ruthenium red procedure. × 35,000.

![Figure 10](image10.png)
**Figure 10** Deposit of the stained material in basal infoldings. Endothelial lining mechanically destroyed. Basement membrane heavily stained (arrows). Ruthenium red procedure. × 35,000.
FIGURE 11 Fragment of a renal arteriole. Lumen, Lu; elastic component of the internal elastic membrane, El. The kidney was perfused with glutaraldehyde-ruthenium red mixture before the routine procedure. Slight deposits of the reaction product are seen on the endothelial lining and in micropinocytotic vesicles of endothelial cells (arrows). × 48,000.

596 THE JOURNAL OF CELL BIOLOGY VOLUME 40, 1969
observed with the colloidal iron method. The reaction product was also seen at the endothelial lining of capillaries (Fig. 15). Moreover, it was noted that mechanical damage to cells resulted in the accumulation of colloidal thorium particles at various cell structures, e.g. the nuclear envelope (Fig. 16 a) and the surface of mitochondria (Fig. 16 b). One could also observe the presence of colloidal thorium particles in the gaps between endothelial or epithelial cells and the detached, underlying basement membrane (Fig. 15).

With regard to the colloidal thorium procedure, a pretreatment with neuraminidase failed to yield positive results.

**Discussion**

A positive reaction at the free cell surfaces of the epithelium of the nephron was obtained with the ruthenium red procedure and with both colloidal metal methods as well. This study has revealed that the extraneous coat of the luminal face of nephron epithelium is a constant feature.

In the ruthenium red procedure, the content of the reaction product in the surface coat varied considerably at various levels of the nephron. In the glomeruli, the surface coat was most marked at the foot processes. In the tubular part of the nephron the intensity of the surface coat depended on the localization of the microvilli. The reaction itself required appropriate pH values for its optimum effects at each particular level of the nephron.

In this procedure the intercellular spaces and the basal membrane infoldings of tubular epithelia were also marked by stained material. At these two sites the reaction, as compared with that at the luminal face of the epithelial cells, was even much stronger. Not only the cell surfaces were
coated, but also the interspaces were filled with stained material. Similar stained granular particles were scattered along the basal surface of epithelial cells. A strongly stained basement membrane was observed only when the endothelial lining of capillaries was mechanically detached. In the ruthenium red procedure the luminal face of capillary endothelial cells was also positive.

At higher magnifications the relation between the ruthenium red-marked material of the surface coat and the cell membrane was evident. The ruthenium red-positive substance seemed to arise from, or to be closely attached to, the outer leaflet of the unit membrane. A similar finding was previously noted in other cell membranes by Luft (17, 18) and also was observed by us in the alveolar lining film of the lungs (13).

The ruthenium red technique enhanced the contrast considerably and preserved distinctly the ultrastructural details of cells.

In the colloidal iron technique the free cell surfaces of the podocytes, proximal and distal convoluted tubules, Henle loops, as well as the endothelial lining of capillaries were regularly stained. The Hale-positive material was located outside the cell membrane. The intercellular spaces were free of reaction product. A light scatter of particles along the basal surface of the cells was observed irregularly.

Staining with colloidal thorium revealed similar results. The free surfaces of cells were regularly stained. A light scatter of thorium particles could be also observed along the basal surface of the epithelial or endothelial lining. Agglomerations of thorium particles were easily observed on the organelles of mechanically disrupted cells, e.g. at the outer membrane of mitochondria and at the nuclear envelope. The reaction product was absent within the intact cells. The localization of metal particles at the organelles of damaged cells could indicate that the particles could be adsorbed unspecifically.

According to Luft (19), the reason for the increased density as seen in electron micrographs after the ruthenium red procedure is that ruthenium red binds to glycoproteins in the tissue and acts as a catalyst in that way, so that one
molecule of ruthenium red reduces several molecules of osmium tetroxide. In the ruthenium red procedure the dye penetrates the intercellular spaces and the basal infoldings of the cell membrane. In these sites the effects of the reaction were strongly marked. This strong reaction is to be contrasted with the rather poorly marked effect of reaction in the case of free cell surfaces. The existing difference could be explained by accumulation of ruthenium red binding material in the interspaces and the basal infoldings as well. A comparatively small amount of reaction product was observed in the basement membrane. This fact could be explained by a poor penetration of dye into the inner part of the basement membrane.

The specificity of the methods recommended for use in the electron microscope technique to render visible the stained material at the cell surfaces calls for a careful and critical interpretation (15, 23).

The fact that Hale staining of the cell surface was removed by neuraminidase treatment might indicate that carbohydrates containing sialic acids were responsible for the effect of the reaction. The obtained results confirm the data reported in literature with respect to the fact that the surface coat consists of glycoproteins containing sialic acid residues (4, 14, 22-24). When one considers the differences in the effects of the various procedures, it seems that even the fact of penetration of dye into the tissue has a great influence on the effect of the reaction itself (16, 26).

In the literature available at present, there is a great amount of data with respect to the functions of the surface coat of the cell membranes (3). The selectivity of these functions seems to be determined by the specific character of the membrane (1, 5, 7, 8, 10). As regards the coat at the free surfaces of the epithelium of the nephron, the glycoprotein layer of the podocytes might trap the macromolecular proteins (14). A thick coat at the villous surface of the convoluted tubules may force the glomerular filtrate to percolate through a column of polysaccharides. Thus, the fractioning of substances provided for reabsorption could take place. In general a carbohydrate macromolecule layer which can retain water may facilitate con-
FIGURE 16a Colloidal thorium technique. Fragment of mechanically disrupted cell. Thorium particles are seen at the nuclear envelope and in the region of it (arrows). X 22,000.

FIGURE 16b Colloidal thorium technique. Fragment of mechanically disrupted cell. Thorium deposit at the mitochondrial surfaces. The endothelial as well as the epithelial side of the basement membrane is traced by thorium particles (arrows). Remnants of endothelial lining, e. X 22,000.

siderably the diffusion of ions and small molecules and secure "transport pathways" in the kidney (22).

All these data provide evidence that the surface coat of the epithelium of the nephron may not only be an important site of some physiological activities at the ultrastructural level but also may play a leading role in the onset and progress of some pathological processes in the kidney.

The authors wish to express their thanks and appreciation to Mr. Tadanori Yoshioka for his capable technical assistance.

This study was supported by a grant from the Committee on Electron Microscopy of the Polish Academy of Sciences.

Received for publication 18 April 1968, and in revised form 29 October 1968.

REFERENCES


4. EMMELOT, P., C. J. BOZ, E. L. BENEDETTI, and P. RUMKE. 1964. Studies on plasma mem-