THE SURFACE STRUCTURE OF CULTURED RABBIT KIDNEY CELLS AS REVEALED BY ELECTRON MICROSCOPY

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SUMMARY

An epithelioid line of rabbit kidney cells (RK 13), in which the distribution of blood group antigen A had previously been investigated by mixed agglutination, was chosen for studies of the structure of the cell surface. Cells were grown in monolayer culture, and thin sections were examined by electron microscopy after fixation in glutaraldehyde and osmium tetroxide and staining of the sections with lead, or uranyl and lead. Cells were also treated with ruthenium red incorporated in the osmium fixative. Other cells were fixed in lanthanum or potassium permanganate, and both stained and unstained sections were examined.

The morphology of RK 13 cell surfaces is described. There is apparently no great degree of cellular specialization. The treatment with ruthenium red resulted in dense staining of a layer of the cell surface that is not visible in conventional preparations, and sometimes in staining of the surfaces of intracellular organelles. Lanthanum permanganate fixation also revealed a dense layer on the surface of the plasma membrane; a less dense surface layer was distinguished in many cells fixed in potassium permanganate.

The reaction of ruthenium red with the cell surface is probably due to the presence of acidic glycoproteins, but the chemical specificity of the staining method is not yet clear. The nature of the material revealed by lanthanum and potassium permanganates is also undefined. However, these staining methods reveal that the cell surface is more complex than is apparent in cells prepared by conventional techniques. The additional surface layer is probably the site of many blood group substances and other compounds involved in the physiological reactions of the cell surface.

INTRODUCTION

Early studies with the electron microscope revealed a trilaminar structure in the plasma membrane of a variety of cells and these observations culminated in the concept of the 'unit membrane' as proposed by Robertson (1959). In the original concept the 3 layers of the unit membrane, 2 outer dense layers and a central less dense layer were all of about the same thickness (about 2·5 nm), but it soon became clear that considerable modifications of this basic structure occur. The total thickness of many membranes is greater than the 7·5 nm originally proposed and the 2 outer dense layers are often of unequal thickness. Furthermore, an additional layer of filamentous material is present on the outer surfaces of many epithelial cells (see, for example, Ito, 1965). The visibility and apparent thickness of these layers of the membrane depend upon the method of fixation and staining used, and as knowledge

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of the chemistry of membranes increased, it became clear that some components of the plasma membrane are not stained, and therefore are not seen in conventional preparations. In particular, the glycolipids and glycoproteins which are important constituents of the surface layers of cells (Watkins, 1967; Cook, 1968) are not revealed.

Two approaches may be taken to demonstrate these components of the membrane; one involves the use of special stains which react with the carbohydrate-containing components of the cell surface, and the other the application of immunochemical staining techniques. Chemical staining techniques have been more widely applied to the cell surface than immunochemical staining techniques, and the work of Rambourg & Leblond (1967) with periodic acid-silver methenamine and colloidal thorium has indicated the widespread distribution of carbohydrate-containing cell surface layers.

The aim of the present study was to investigate the surface structure of a plasma membrane in detail using these 2 methods. A line of rabbit kidney cells (RK 13) was chosen for examination, since the distribution and expression of a glycolipid, the blood group antigen A, was already well known from the studies of Franks & Dawson (1966) with the mixed agglutination technique.

This paper describes the fine structure of RK 13 cells prepared by routine techniques and after the application of 2 special stains, ruthenium red and lanthanum permanganate. Particular emphasis is placed on the structure of the surface layers, but a short description of the fine structure of the other components of the cells is also included. The results of immunochemical studies on the same cells will be described in a subsequent paper (Dimmock, Franks & Glauert, in preparation).

MATERIALS AND METHODS

Cells

Cultures of an epithelial line of rabbit kidney cells (RK 13) were obtained from Dr D. Franks of the Department of Pathology, University of Cambridge, in 1966; the cells were originally obtained by Dr Franks from Dr G. Christofinis (Dawson & Franks, 1967). The early history of the line is described by Christofinis & Beale (1968).

Media

The cells were grown in NCTC 109 medium or in 199 medium supplemented with presuckling or foetal calf serum. The calf serum was tested to ensure that only sera lacking J substance (the cattle equivalent of blood group A substance) were used in making medium. The absence of J substance from the medium is important in immunological studies on the distribution of blood group A substance on cell surfaces (Dimmock, Franks & Glauert, in preparation) since it is known that serum components can be adsorbed on to cells (see Coombs, Daniel, Gurner & Kelus, 1961).

NCTC 109 medium was obtained from Dr D. Franks or from General Biochemicals; that supplied by Dr Franks was buffered with a tris-citrate buffered balanced salt solution and contained certain supplementary sugars (Franks & Dawson, 1966). Medium 199 was made up from a liquid concentrate obtained from Flow Laboratories.

Cell culture

The cell line was grown in soda glass bottles at 37.5 °C as described by Franks & Dawson (1966). Before subculture the cell layer was rinsed with 0.0005 M EDTA in solution (a) of Dulbecco & Voor's (1954) phosphate-buffered saline solution (which will subsequently be re-
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ferred to as saline), and the cells were subcultured after treatment with 0.25% trypsin (Difco 1:250). The cultures were gassed after treatment with a mixture of 5% CO₂ + 20% O₂ + 75% N₂ when General Biochemicals NCTC 109 medium was used. Cultures were grown until the cell population was confluent (3–7 days), and the medium was changed when the pH fell below pH 7, or after 3 or 4 days.

Culture of cell layers for electron microscopy

Cells can be prepared for electron microscopy by trypsinization and centrifugation but this method was rejected for the present experiments since trypsin is known to remove sialomucoprotein from cell surfaces (Cook, Heard & Seaman, 1960). As an alternative approach many different substrata have been used beneath cultured cells, including carbon (Robbins & Gonatas, 1964; Barnicot & Huxley, 1965), collagen (Heyner, 1963), collodion (Meiselman, Kohn & Danon, 1967), and Millipore filters (Dalen & Nevalainen, 1967; McCombs, Benyesh-Melnick & Brunschwig, 1968). Several substrata were tested, but it was found that layers of glutaraldehyde-fixed RK 13 cells could easily be removed from glass surfaces, and since the orientation of the cell layer in the block was not of crucial importance the cells were routinely prepared for electron microscopy by growth directly on glass coverslips. In this way cells for both light and electron microscopy could be grown under identical conditions.

The layer of cells was removed from the glass surface with a razor blade either before osmium tetroxide fixation but after glutaraldehyde fixation, or in 70% ethanol during the course of dehydration (Daniel, Dingle, Glauert & Lucy, 1966). The layer was thereafter treated as a piece of tissue. After embedding it was always possible to select areas where the cells could be sectioned at right angles to the plane of the layer.

Preparation of cell layers for electron microscopy

For routine examination the RK 13 cells on coverslips were usually rinsed in saline at room temperature and then placed in either cacodylate- or phosphate-buffered glutaraldehyde in Columbia staining jars at 4 °C or at room temperature. A 25% aqueous stock solution of glutaraldehyde was diluted 1 in 10 with 0.1 M cacodylate buffer, pH 7.2, or with 0.1 M phosphate buffer, pH 7.4. The cacodylate buffer contained 10 mM calcium chloride and the phosphate buffer contained a small amount of calcium from calcium chloride added in drops as suggested by Palfrey & Davies (1966). After fixation for 30 min the coverslips with the cell layers were rinsed in the appropriate 0.1 M cacodylate or phosphate buffer 2 or 3 times for at least 30 min for each change of buffer. Sometimes the cells were stored in buffer overnight; they were rinsed at either 4 °C or at room temperature and storage was always at 4 °C. Subsequently the cells were fixed in buffered osmium tetroxide for 30 min at 4 °C or at room temperature. Zetterqvist's buffered osmium tetroxide (quoted by Glauert, 1965a) was used for cells fixed in cacodylate-buffered glutaraldehyde and phosphate-buffered osmium tetroxide (1% osmium tetroxide in 0.1 M buffer, pH 7.4, with calcium chloride) was used for cells fixed in phosphate-buffered glutaraldehyde. Cell layers were then dehydrated in ethanol and if they had not been removed from the coverslips before osmium tetroxide fixation, they were removed in 70% ethanol. The cell layer was soaked in propylene oxide and embedded in Araldite in gelatine capsules. The Araldite was cured at 60 °C for at least 24 h.

Thin sections were cut on an A. F. Huxley microtome or on an LKB Ultrotome III and were stained with either 1–2% aqueous uranyl acetate (uranil) for 30 min (Glauert, 1965b) and/or lead citrate (lead) (Venable & Coggeshall, 1965) for 2–10 min (usually 5 min). The sections were examined in an EM 6B electron microscope operating at 80 kV with a 50–70 m aperture.

Phase-contrast microscopy

 Cultures of RK 13 cells which had not reached confluence were fixed in glutaraldehyde and examined by phase-contrast microscopy with a Reichert Zetopan microscope fitted with both positive and negative (anoptral) phase-contrast objectives.
Staining methods for cell surfaces

Ruthenium red. A slight modification of Luft's technique of staining with ruthenium red (Luft, 1966b; Pate & Ordal, 1967) was applied to RK 13 cells. Cells were fixed in phosphate-buffered glutaraldehyde and rinsed in phosphate buffer. They were then fixed for 3 h at room temperature in a freshly prepared solution containing both osmium tetroxide and ruthenium red which had the following composition: 0.2 M phosphate buffer (pH 7.4, containing calcium chloride), 0.5 ml; 2% aqueous osmium tetroxide, 0.5 ml; 0.2% aqueous ruthenium red, 0.5 ml. Control sets of cells were exposed to the standard phosphate-buffered osmium fixative for 30 min (the usual fixation period) or 3 h. The specimens were subsequently dehydrated and embedded as previously described. The cell layers were scraped off the coverslips either before treatment with ruthenium red or in 70% ethanol. Sections were examined after being stained with lead or with uranyl and lead, or without staining.

Lanthanum permanganate. RK 13 cells were fixed by the method of Lesseps (1967), and potassium permanganate fixation was used as a control. A lanthanum permanganate solution was prepared as described by Lesseps (1967). Stock solutions of veronal-acetate (2.49 g of sodium veronal and 1.15 g of anhydrous sodium acetate dissolved in distilled water to give a volume of 100 ml) and of Ringer's salt solution (8 g sodium chloride, 0.4 g potassium chloride and 0.2 g calcium chloride dissolved in distilled water to a volume of 100 ml) were prepared. The lanthanum permanganate was prepared from lanthanum nitrate trihydrate and potassium permanganate (0.25 g of each salt) dissolved in 5 ml of the stock veronal-acetate solution and 1.5 ml of the Ringer's salt solution. The resulting solution was titrated to pH 7.8 with 0.1 N hydrochloric acid and the volume made up to 25 ml with distilled water. The potassium permanganate fixative was prepared by dissolving 0.25 g of potassium permanganate in 5 ml of the stock veronal-acetate solution and 1.5 ml of the Ringer's salt solution. The resulting solution was titrated and made up to volume in the same way as the lanthanum fixative.

The cells were rinsed in phosphate-buffered saline or in isotonic Ringer's solution and were fixed in the cold (4 °C) in lanthanum or potassium permanganate fixative for 1 h; they were then rinsed 3 times in phosphate-buffered saline or Ringer's solution, dehydrated and embedded. Alternatively, cells were rinsed in phosphate-buffered saline or Ringer's solution, fixed in cacodylate-buffered glutaraldehyde and then washed in cacodylate buffer before being fixed in lanthanum or potassium permanganate fixative for 1 h. The cells were removed from the coverslips in 70% ethanol. Sections were examined after lead staining, uranyl and lead staining, or without staining.

RESULTS

Cells prepared by conventional methods

RK 13 cell cultures examined by phase-contrast microscopy resembled those used by Franks & Dawson (1966). The fine structure of a cell is shown in Fig. 1. RK 13 cells show no particular specialization.

Cell membrane. In cells fixed in glutaraldehyde and osmium tetroxide and stained, after sectioning, with lead or uranyl and lead stains, the cell membrane usually appears as a single dark line a little less than 10 nm wide (Fig. 2) but in a very few micrographs it is seen as a triple-layered unit membrane, the 3 layers being of approximately equal thickness (Fig. 3). In such conventionally fixed cells there is no thickening of the outer layer of the unit membrane as can be demonstrated by special staining methods. In many cells a layer of fibrils is seen beneath some regions of the plasma membrane (Fig. 2,f).

Other surface features. Small depressions in the surface membrane are seen in some cells. These vary in shape from shallow depressions to flask-shaped pits (Fig. 4); a few appear to be completely detached from the cell surface and to be intracellular
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(Fig. 4, arrow). The cytoplasmic surface of the membrane bounding these depressions is slightly denser than that of the adjacent membrane (Fig. 4). These structures resemble the 'coated vesicles' described by many authors (see Fawcett, 1965).

Fine projections representing microvilli and surface folds are present on the upper surfaces of most RK13 cells and occasionally the microvilli are branched. Many microvilli are associated with a basal vesicle 60–160 nm in diameter; this may be an intracellular structure, but in some micrographs the vesicle is obviously open to the surface. The structure of the membrane of these vesicles resembles that of the coated vesicles described above. A similar association of open vesicles with the bases of microvilli has been described by Yamada (1955) in the epithelial cells of the mouse gall bladder.

Cell-to-cell junctions. RK13 cells in a monolayer abut on each other with a roughly vertical or sloping junction (Fig. 5). The surfaces of the cells at these junctions are characterized by long processes, and frequently the processes of neighbouring cells interdigitate (Fig. 5). Some of these processes may be folds, since they lie parallel to each other for quite long distances (Fig. 5), but circular profiles are seen, indicating that microvilli are also present. In regions where the processes are absent the intercellular gap is often constant in width for long distances.

'Cilia'. In a few micrographs of cells from one culture structures somewhat resembling cross-sections of cilia were seen (Fig. 6); they were unusual in having a ring of only 7 double fibrils around the periphery and 2 double fibrils in the centre. There are many references in the literature to unexpected and atypical cilia (Barnes, 1961; Sorokin, 1962; Hando, Okada & Zamboni, 1968). It seems unlikely that these cilia have any functional significance.

Cells stained with ruthenium red

During fixation with osmium tetroxide solution containing ruthenium red, the cell layer turns a dark purplish brown colour. The ruthenium red has no adverse effect on the cohesiveness of the monolayer, which behaves in the same way as monolayers treated with osmium tetroxide without ruthenium red.

Cells fixed in osmium tetroxide have reasonable contrast, which permits the examination of sections to which no stain has been applied. Such sections were examined, in addition to those stained with lead, to define the contribution to the contrast made by the ruthenium red and also to ascertain whether the application of a section stain increased the width of the cell surface layer. It was found that lead staining increases the general contrast but does not alter the width of the cell surface layer.

Cell surface. After ruthenium red staining a very dense granular layer of stain is seen along the surfaces of the cells (Fig. 8). The thickness of the layer varies considerably at different points on the surface of a single cell, and is usually greater on the upper than on the lower surface, which is sometimes almost unstained (Fig. 8) but sometimes stained; it appears that the undersurfaces of cells removed from the cover-slip before treatment with ruthenium red are stained, but that those removed after treatment are not stained. Cells are found which have almost no stain on either surface.
The unit membrane is very clearly visible in cells treated with ruthenium red when the membrane is sectioned at a favourable angle (Fig. 7). The staining of the membrane is asymmetrical in most micrographs, the inner dense leaflet being narrower than the outer dense leaflet. Some measurements were made but it is realized that the thickness of the various layers of the membrane measured on micrographs cannot be very accurate for several reasons, including variations between the actual and the nominal magnifications of the electron microscope, alterations in the dimensions of the prints during processing, and the inaccuracies inherent in measuring very small distances. Measurements of the inner leaflet of the unit membrane indicate that it is usually of the order of 5 nm wide (Fig. 7) but in some micrographs it is wider. The pale central leaflet is somewhat narrower than the inner dense one. The outer leaflet shows the greatest variation in width, being similar to the inner one in some cells, 2 or 3 times wider in most cells, and very much wider in others.

The membranes of the microvilli appear similar to the rest of the cell membrane. Coated vesicles are occasionally seen, but not in regions where the membrane is very densely stained; it is not clear whether they are obscured by the stain or are absent in these regions. Cell-to-cell junctions are not conspicuously different in monolayers treated with ruthenium red from those seen in controls. The 2 adjacent membranes are stained but there is no intercellular staining.

**Cytoplasm.** The cytoplasm of some cells is stained by ruthenium red (Fig. 8) but that of others is not (Fig. 7). It has been suggested that ruthenium red does not cross intact membranes, (Luft, 1966b), and it is noticeable that small breaks are more often seen in the plasma membranes of *RK 13* cells whose cytoplasm is stained by ruthenium red (Fig. 8). The internal staining which results from this penetration sometimes shows a gradient from intense staining near the surface to none further in (Fig. 8). Ruthenium red stains a surface layer on the membranes of mitochondria, cisternae of the endoplasmic reticulum, elements of the Golgi apparatus, and the nucleus (Fig. 8). In general only the outer surfaces of these membranes appear to be stained.

**Cells fixed in permanganate fixatives**

During the fixation of monolayers of *RK 13* cells with solutions of potassium or lanthanum permanganate the cell layer turns a deep golden brown. Permanganate-fixed cell layers are very fragile and tend to fragment as they are removed from the coverslip, and consequently there is a high proportion of damaged cells in the material examined by electron microscopy. However, the upper cell surface, which is of primary importance in these studies, is relatively undisturbed. Glutaraldehyde fixation was used before permanganate fixation in an attempt to stabilize the monolayer; it had a slight effect, but the cell layer still disintegrated rather easily. This fragility is probably due to the action of the permanganate, since cell layers fixed in glutaraldehyde but not post-fixed in osmium tetroxide were as stable as those fixed in glutaraldehyde and post-fixed in osmium tetroxide. The embedding method used in this study is not very satisfactory for permanganate-fixed material. *RK 13* cells fixed in permanganate fixatives have a very different appearance from those fixed in glutaraldehyde and osmium tetroxide. The major differences are the
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extreme electron density of the membranous components of the cell, and the com-
parative lack of other cytoplasmic and nuclear detail; the resulting impression is of
very conspicuous membranes set against a rather featureless background. Glutaralde-
hyde prefixation does not radically alter the appearance of the cells, but it preserves
more cytoplasmic and nuclear detail than fixation in permanganate alone.

The contrast given by potassium or lanthanum permanganate alone was sufficient
for electron microscopy but staining with lead, or uranyl and lead, considerably
enhanced the contrast.

Cell surface. The plasma membrane is a very conspicuous feature of cells fixed in
permanganate solutions. In RK 13 cells fixed in potassium permanganate the plasma
membrane is very occasionally seen as a trilaminar structure (unit membrane) (Fig.
10), slightly more than 9 nm wide, in which the width of each leaflet is of the order
of 3 nm. It more often appears as a single dense line 8-5–17 nm wide. Exact measure-
ments are difficult to make since the plasma membrane seems to be asymmetrical,
with a dense clearly defined inner surface and a less dense and less clearly defined
outer surface. In many cells this ill-defined outer surface can be distinguished as a
separate layer which in most places is up to 35 nm in width and which occasionally
extends from the cell membrane for as much as 1 μm as diffuse amorphous material
(Fig. 9, arrow). It may be a chemical precipitate formed during fixation, but this is
unlikely since the coverslips were placed vertically and were washed after fixation by
filling and emptying of the Columbia jars. More probably the material is derived from
some substance released from damaged cells which is sufficiently sticky to remain
attached to the cells during washing. It is also remotely possible that the amorphous
material represents a genuine, if irregular, component of the cell surface.

The plasma membranes of cells fixed in lanthanum permanganate solution are
densely stained and frequently appear trilaminar. In one experiment, however, a
different result was obtained; the membrane never appears trilaminar and the whole
structure is very dense indeed. The inner surface is more clearly defined than the
outer one which is covered with dense ‘whiskers’. The depth of the dense structure is
17-42-5 nm (Fig. 11).

In all other experiments the trilaminar structure of the membrane was seen fairly
frequently (Fig. 12); the inner dense and the central less-dense layers are about
3-3 nm wide, whereas the outer dense layer varies considerably from as little as
3-3 to nearly 100 nm wide. In some places the outer layer appears to have 2 com-
ponents, an internal dense layer and a slightly less dense, irregular or fluffy layer on
the outer surface (Fig. 12), but this distinction is not always apparent. Doggenweiler
& Frenk (1965) illustrate, and Lesseps (1967) describes and illustrates, these 2 com-
ponents of the outer layer of the plasma membrane of retinal rods and dissociated
embryonic chick cells.

The plasma membranes of the microvilli are similar to the adjacent plasma
membrane. Coated vesicles are not a conspicuous feature of the cell surface of
permanganate-fixed RK 13 cells, but are occasionally seen.

Prefixation in glutaraldehyde has little if any effect on the appearance of the plasma
membranes of cells fixed in permanganate fixatives.
Cell-to-cell junctions are similar in morphology to those between cells fixed with glutaraldehyde and osmium. A very dense deposit is sometimes present between lanthanum-fixed cells; in some regions it fills the whole intercellular gap, but elsewhere it occupies only the gap where the cells are closely apposed, leaving the wider spaces empty with only the membranes stained. In other micrographs the dense deposit seems to be absent.

**DISCUSSION**

This study has shown that the plasma membrane of RK 13 cells prepared by conventional means usually appears as a single dense line in favourably oriented sections; the trilaminar ‘unit membrane’ is rarely observed after fixation in glutaraldehyde and osmium tetroxide and section staining with lead or with uranyl and lead. Similar results have been reported for a variety of tissue cells, including kidney cells, by Farquhar & Palade (1963); these authors concluded that the outer dense layer of the membrane is not stained by this routine technique. The outer layer is clearly visible, however, when cells are stained in block with uranyl acetate before dehydration (Farquhar & Palade, 1963, 1965; Marchesi & Palade, 1967).

The unit membrane structure is observed frequently in the plasma membranes of cells stained with ruthenium red, and in addition a further densely stained layer is observed on the outer surface of the membrane. It is clear therefore that ruthenium red reveals a surface layer of the membrane that is invisible in preparations fixed and stained by routine techniques. A similar layer has been observed on the surface of a variety of cells after staining with ruthenium red (Luft, 1964, 1966a, b; Kelly, 1966; Martinez-Palomo & Brailovsky, 1968; Brooks, 1969).

The staining properties of ruthenium red in the presence of osmium tetroxide are not yet understood, but there is some evidence that it reacts with acidic polysaccharides and with certain other acidic components (Luft, 1966b). The staining mechanism may not be a simple reaction between the ruthenium red and a component of the cell surface, and consequently there may be a disparity between the extent of the visible deposit of stain and the amount of carbohydrate-containing material at the surface of the membrane. Until more is known of the chemical processes by which the deposit is formed, it would be unwise to draw definite conclusions about the morphology of the cell surface from observations with ruthenium red, although such attempts have been made (Martinez-Palomo & Brailovsky, 1968). It is possible to conclude, however, that the presence of a layer of material on the surface of the membrane can be reliably detected by this staining method. Furthermore, other techniques such as mixed agglutination (Franks & Dawson, 1966) and cell electrophoresis (Cook, 1968) have indicated the existence of glycolipids and glycoproteins on the outer surface of cell membranes, and it seems reasonable to conclude that these components are present in the layer stained with ruthenium red. The possibility that other components contribute to the staining reaction cannot be ruled out, however.

The inner dense layer of the unit membrane structure of the plasma membrane of RK 13 cells is also stained by ruthenium red. This staining is seen in some cells into
which ruthenium red does not appear to have penetrated, suggesting that the stain is capable of reacting with material in both dense layers of the intact membrane; it is probable that minute breaks are present and that ruthenium red has access to the inner surface of the membrane through these.

The gradient of staining seen in cells penetrated by ruthenium red probably results from the slow penetration of the stain (Luft, 1966b).

Two different appearances were obtained in RK 13 cells after lanthanum permanganate fixation. An extremely dense, whiskery layer was seen in one experiment and resembled the layer illustrated by Lesseps (1967) in his micrograph of the surface of dissociated heart ventricular cells. It is possible that the extreme density of this layer is due not only to the reaction of lanthanum with components of the cell surface, but also to the presence of anions that can form insoluble salts with lanthanum. The anions carbonate and phosphate were present in the Tyrode and Hanks's salt solutions used by Lesseps; and phosphate in the phosphate-buffered saline used by the author in the one experiment in which this type of staining was found, and they may have reacted with the lanthanum. For this reason Ringer's salt solution, which contains no anions but chloride, and cacodylate buffer, which contains no anions but cacodylate and chloride, were used in the other experiments; a less dense and more irregular layer was then observed on the surfaces of lanthanum permanganate fixed cells.

The nature of the material that stains with lanthanum was investigated by Lesseps (1967) and his results indicate that the material is sensitive to the action of phospholipase C, suggesting that it may contain phospholipids. Lesseps (1967) discusses the possibility that substances other than phospholipid are present in the material stained by lanthanum. Lanthanum may well stain the same material that is made visible when cells are treated with uranyl acetate before dehydration; this treatment stains the outer dense layer of the unit membrane (Farquhar & Palade, 1963, 1965) and also reduces the extraction of phospholipid phosphorus (Silva, Carvalho Guerra & Magalhães, 1968) from specimens during preparation for electron microscopy.

Overton (1968, 1969) has used a different technique for staining cell surfaces with lanthanum and concluded that the stained material breaks down when treated with EDTA, trypsin or pronase. It seems clear that the mechanism of staining depends upon the technique used and that a careful chemical study of the formation of deposits containing lanthanum compounds in the complex physicochemical environment of the cell surface will be necessary before the full significance of lanthanum staining is understood.

In conclusion, this study of the surfaces of RK 13 cells has indicated the existence of an outer layer containing a compound or compounds that react with ruthenium red and lanthanum permanganate. It seems probable that the layer contains acidic glycoproteins and possibly phospholipids. This layer is of great physiological importance since it is the part of the cell that is in direct contact with the environment; and the components of the cell surface that are concerned with such properties as cell recognition, contact inhibition and antigenicity, may well be present in this layer.
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Fig. 1. Survey micrograph of an RK 13 cell sectioned perpendicularly to the plane of the substratum, showing the humped region containing the nucleus. The Golgi zone (g) lies near the nucleus, and mitochondria (m) and cisternae of the endoplasmic reticulum (er) are scattered through the cytoplasm. A single nucleolus (n) is visible. Fixation in phosphate-buffered glutaraldehyde and osmium tetroxide; section stained with lead citrate; x 7500.

Figs. 2, 3. The cell membrane of two RK 13 cells. In Fig. 2 the membrane is seen as a single dark line, whereas in Fig. 3 the unit membrane pattern is visible. In Fig. 2 fibrils (f) are visible beneath the plasma membrane. Both cells fixed in phosphate-buffered glutaraldehyde and osmium tetroxide; sections stained with lead citrate. x 60000.

Fig. 4. Coated vesicles of various shapes are visible along the surfaces of 2 adjacent cells. One vesicle (arrow) is apparently detached from the surface. Fixed in phosphate-buffered glutaraldehyde and osmium tetroxide; section stained with lead citrate. x 60000.
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Fig. 5. Cell-to-cell junction between two RK 13 cells with numerous interdigitating processes. Fixation in phosphate-buffered glutaraldehyde and osmium tetroxide; section stained with lead citrate. × 30000.

Fig. 6. Section of part of an RK 13 cell in which a 'cilium' is present. (The small black dots on the cell surface are ferritin.) Fixation in phosphate-buffered glutaraldehyde and osmium tetroxide; section stained with lead citrate. × 60000.

Fig. 7. The upper surface of an RK 13 cell stained with ruthenium red. The cytoplasm appears to be unstained, whereas the surface is heavily and irregularly stained, and the unit membrane pattern is clearly visible. Fixation in glutaraldehyde and osmium tetroxide solution containing ruthenium red. Section not stained. × 60000.
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Fig. 8. Section of an RK 13 cell stained with ruthenium red. A thick granular layer of stain is present on the upper but not on the lower surface of the cell. Staining of some internal organelles has occurred and is more pronounced near the upper surface of the cell. Fixation in phosphate-buffered glutaraldehyde and osmium tetroxide solution containing ruthenium red. Section not stained. × 30000.
Fig. 9. Surface of an \textit{RK13} cell fixed in potassium permanganate. Loose fluffy material is visible attached to and extending away from the cell membrane (arrow); section stained with lead citrate. \( \times 24,000 \).

Fig. 10. An \textit{RK13} cell fixed in potassium permanganate; section not stained. The unit membrane pattern is visible. \( \times 120,000 \).

Fig. 11. Surface of a cell fixed in lanthanum permanganate, showing a very dense deposit; section stained with lead citrate. \( \times 60,000 \).

Fig. 12. Surface of an \textit{RK13} cell fixed in lanthanum permanganate but with a less-dense surface layer than that shown in Fig. 11; section stained with lead citrate. \( \times 60,000 \).
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