THE REMOVAL BY PHOSPHOLIPASE C OF A LAYER OF LANTHANUM-STAINING MATERIAL EXTERNAL TO THE CELL MEMBRANE IN EMBRYONIC CHICK CELLS

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ABSTRACT

Fixation of embryonic chick cells (heart, neural retina, and limb bud) in the presence of lanthanum ions shows the presence of an electron-opaque layer, about 50 A thick, external to the cell membrane. This layer, designated LSM (for lanthanum-staining material), is not removable by trypsin, pronase, EDTA, DNase, α-amylase, neuraminidase, or *N*-acetyl-L-cysteine. However, phospholipase C, in concentrations as low as 0.001 mg/ml, succeeds in stripping the LSM from the cell surface. Heating the enzyme preparation does not inhibit this activity, but removal of divalent cations does; both of these results are consistent with the known properties of phospholipase C. The LSM is present at the cell surface in the control tissues and on cells dissociated from the tissues by proteolytic enzymes and EDTA. These results are interpreted to mean that the LSM is probably an integral part of the cell and not an extraneous coat. How this phenomenon bears on the problem of cellular adhesion is discussed, as is the possible chemical composition of the LSM.

INTRODUCTION

Most electron micrographs of animal tissues show an electron-transparent region 100–200 A wide between apposed cells. This "gap" is often considered to be filled with extracellular material. If this interpretation is correct, the adhesions between cells are formed either by this extracellular material itself (26) or by forces capable of bridging this large (in molecular dimensions) "gap", such as London-van der Waals attractive forces (9, 10). But if this material external to the plasma membrane is a part of the cell and forms its outermost surface, then the bonds between cells could be formed directly by short-range interaction of molecules in these adjacent surface layers (21, 29). According to the elegant analysis by Steinberg (34, 35), quantitative variation in the number of adhesive sites would be sufficient to explain many aspects of the intricate morphogenetic movements occurring during animal development. A recent review by Trinkaus (36) stresses the importance of learning more about the properties of the cell surface in connection with the problem of morphogenetic movements.

Doggenweiler and Frenk (11) have developed a technique for heavily staining the material external to the membrane at the surface of cells. This technique involves treating the cells with lanthanum ions either prior to or during the fixation of the cells for electron microscopy. Karnovsky and Revel (18) have used a modification of this tech-

nique and achieved the same kind of intense staining.

Without the use of this technique the material external to the plasma membrane is so electron-transparent that previous attempts on our part to study the effects of various enzymes upon it were unsuccessful (20). The findings of Doggenweiler and Frenk (11) reopened the possibility of detecting effects of various agents upon the cell surface. Therefore, before fixing the cells with a slight modification of their technique, we treated cells with several different enzymes and chemical agents in the hope of gaining some knowledge of the chemical composition and biological role of this material at the cell surface.

The term *unit membrane* will not be used in this paper because of the criticism recently leveled against this concept (5, 14). Electron micrographs usually picture at the cell periphery a triple-layered structure consisting of two electron-opaque lines separated by an electron-transparent line. This triple-layered structure will be referred to simply as the plasma or cell membrane.

MATERIALS AND METHODS

Heart ventricles and anterior limb buds from 5-day-old chick embryos (White Leghorn) and neural retina from 7-day-old embryos were cut into small pieces in Hanks' solution, pH 7.4. Some of the tissue fragments were then transferred directly into the fixative. The other tissue fragments of ventricles or neural retinas were dissociated into a suspension of single cells as follows. The tissue pieces were incubated on a rotating drum for 15 min at 37°C in a Ca- and Mg-free Tyrode's (CMFT) solution containing 3% trypsin 1:250 (Difco Laboratories, Detroit, Mich.), 1% pancreatin 4 × USP (Nutritional Biochemical Corporation, Cleveland, O.), and 0.12% disodium ethylenediaminetetraacetate (EDTA), adjusted to pH 7.6. This solution was replaced by Ca-free Tyrode's solution containing 0.1 mg/ml crystalline deoxyribonuclease (DNase, Worthington Corporation, Harrison, N.J.), and the tissues were dissociated by touching the tube briefly to an Adams Cyclomixer so that the contents (about 1 ml) were rapidly swept in a thin layer around the walls of a 17 ml glass tube. The cell suspension was divided into aliquots in 3-ml centrifuge tubes, washed once with Hanks' solution, and then either fixed or exposed to further treatment before fixation. About 30 ventricles or 14 neural retinas were used in each dissociation procedure.

Pronase (B grade) (Calbiochem, Los Angeles, Calif.) at a concentration of 1 mg/ml in Ca- and Mg-free Hanks' solution with 0.12% EDTA adjusted to pH 7.5 was used to dissociate one batch of neural retinas.

The following reagents were used in different experiments for the treatment of trypsin-dissociated cells before fixation:

1. α-amylase (Sigma Chemical Co., St. Louis, Mo.) (from *Bacillus subtilis*, crystallized), Type II, 1 mg/ml in Tyrode's solution, pH 6.9.

2. Neuraminidase (General Biochemicals Div., North American Mogul Products Co., Chagrin Falls, O.) (from *Vibrio cholerae*), 1 ml containing 500 units was diluted with 4 ml of Tyrode's solution, pH 7.4.

3. Phospholipase C (phosphatidylcholine cholinephosphohydrolase EC 3.1.4.3, Worthington, from *Clostridium welchii*), several different concentrations and conditions: (*a*) 5, 0.5, 0.1, 0.01 and 0.001 mg/ml in Tyrode's solution, pH 7.4; (*b*) 0.1 and 0.01 mg/ml in CMFT plus 0.12% EDTA, pH 7.4; (*c*) 0.01 mg/ml in Tyrode's solution, pH 7.4, heated for 10 min at 60°C before use.

4. *N*-acetyl-L-cysteine (Baltimore Biological Laboratories, Baltimore, Md), 0.5% and 0.05% in CMFT plus 0.12% EDTA, pH 7.5.

The dissociated cells were kept in the α-amylase and neuraminidase solutions at 37°C for 30 min, and in the *N*-acetyl-L-cysteine solutions at room temperature for 30 min. The dissociated cells were exposed to phospholipase C (PHL-C) at 37°C for 10 min, except in two cases of 20 min exposure to this enzyme, which will be mentioned in the Results section.

The fixative used in these experiments was modified from that developed by Doggenweiler and Frenk (11). Instead of preincubating the cells in 1% La $(NO_3)_3$ or fixing them in $La(MnO_4)_3$, synthesized from $La_2(SO_4)_3$ and $Ba(MnO_4)_2$, as called for in their methods, we fixed the cells and control tissue pieces for one hour at 0°C in a solution of 1% La-$(NO_3)_3 \cdot 6H_2O$ plus 1% $KMnO_4$. The methods of Doggenweiler and Frenk were tried in the earlier stages of this work, but the modified fixative was simpler and yielded identical results. The exact formulation of the modified fixative is as follows: $La(NO_3)_3 \cdot 6H_2O$, 1 g; $KMnO_4$, 1 g; Veronal-acetate buffer (28), 20 ml; Zetterqvist-salt solution (28), 6 ml; 0.1 N HCl, x ml to pH 7.8; Glass-distilled H_2O, to 100 ml total volume. After fixation the specimens were treated with several changes of Tyrode's solution, dehydrated through a graded series of ethyl alcohols, and embedded in Araldite 502 (22). Sections were cut with glass knives on an LKB Ultrotome, collected on carbon-coated grids, and examined in an RCA EMU-3F electron microscope.

RESULTS

In the limb bud, heart and neural retina which have been treated with lanthanum ions, the cell surfaces at or near the edge of every tissue frag-

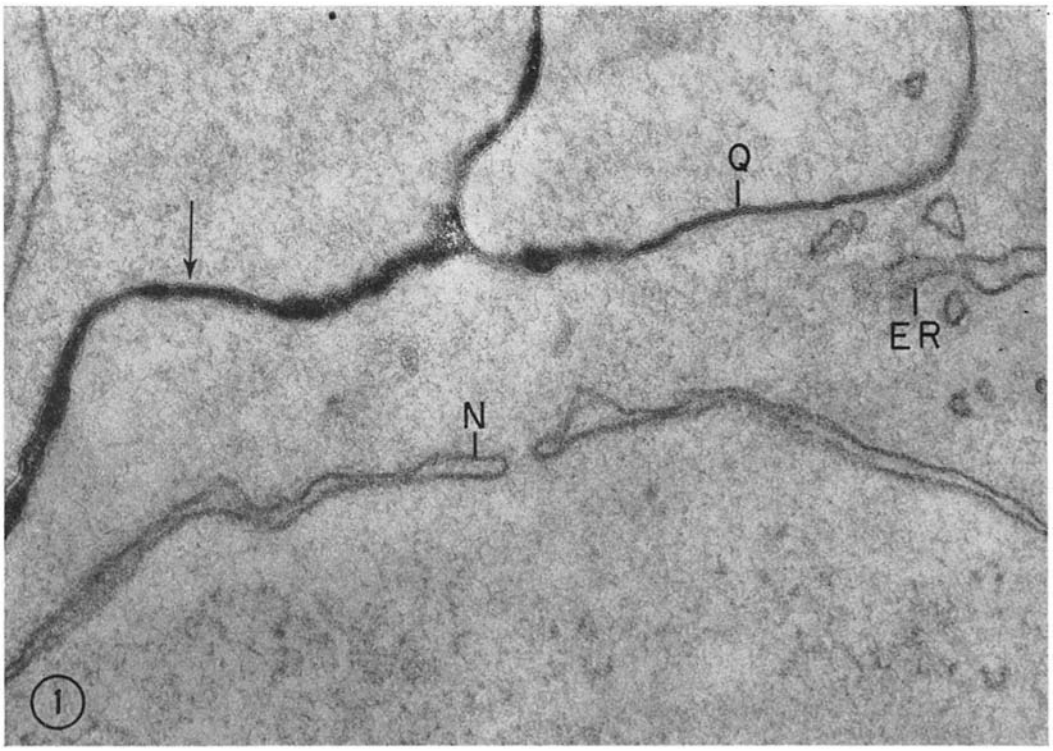

FIGURE 1 Undissociated limb bud cells. The space between the adjacent cell membranes is filled with LSM except in areas where the cell membranes form a quintuple-layered structure (*Q*). The inner dark leaflet of the cell membrane is visible (arrow), but the outer dark leaflet is indistinguishably merged with the LSM. Nuclear membranes (*N*) and endoplasmic reticulum (*ER*) do not show any lanthanum staining. × 83,000.

ment show a thick, electron-opaque layer external to the cell membrane and continuous with the outermost dark leaflet of the membrane (Fig. 1). This lanthanum-staining layer external to the cell membrane will be referred to throughout this paper as the LSM (for lanthanum-staining material). Deeper within the tissue fragments, the cell surfaces present exactly the same picture as they do after conventional permanganate fixation without lanthanum. This absence of staining deeper in the tissue fragments is probably due to lack of penetration of the lanthanum ions, since the LSM appears in the outer layers of all tissue fragments and completely surrounds all cells which were fixed as a suspension of single cells (see below). The LSM and the external dark leaflet of the cell membrane have no separation visible between them, but rather appear as one uniformly electron-opaque layer. The inner dark layer and the light layer of the plasma membrane have the normal widths of about 25 A each. The lanthanum stain does not appear on any membranous structures within the cells; the mitochondria, endoplasmic reticulum, and nuclear membranes, all appear the same as they do after normal permanganate fixation. The explantion for this may be that the La^{+++} does not enter the cell, just as it does not penetrate deeply into tissues. Sections through infoldings of the cell surface do show the same dark layer of LSM found at the cell surface (Figs. 3 and 7).

Since the proteolytic enzymes trypsin and pronase are effective in freeing single cells from tissues (25, 15), it seemed possible that cells treated with these enzymes before fixation might not have the electron-opaque layer external to the cell membrane. But such is not the case. The LSM is consistently found at the surfaces of all trypsin- or pronase-dissociated cells (Fig. 2). Every dissociated cell is bounded by a normal, inner,

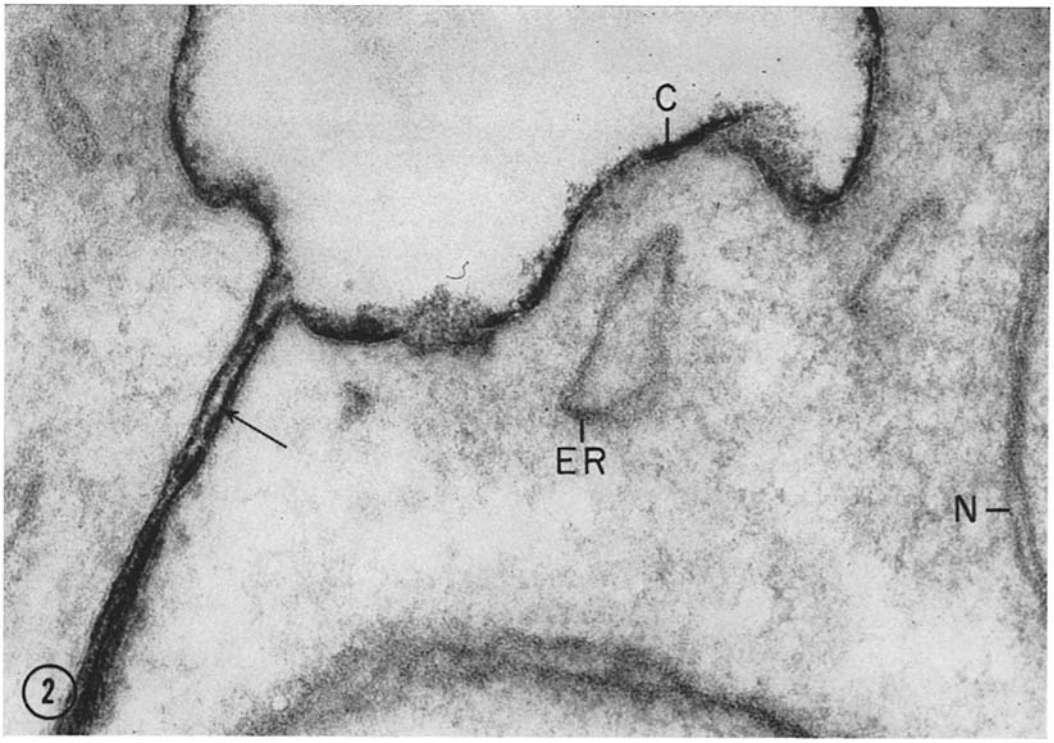

FIGURE 2 Neural retina cells, dissociated from the tissue by trypsin-EDTA. The two cells are still joined together along the surfaces running toward the bottom left corner of the electron micrograph. The LSM has not been removed by the dissociation procedure, but is still present at the exposed cell surfaces (*C*) and at the loosened cell junction, where a narrow light space partially separates the LSM of the two cells (arrow). The inner and middle layers of the cell membrane are visible; the outer layer is continuous with the LSM. No lanthanum stain is present on the nuclear membrane (*N*) or endoplasmic reticulum (*ER*). × 137,000.

dark line, a normal, middle, light line, and a 75–100 A wide outer, dark line, external to which is a fuzzy zone of varying width. If the normal, outer, dark line (25 A wide) is present in the 75–100 A wide outer layer, as indicated by evidence presented below, then the width of the LSM is 50–75 A. In one experiment, to ensure the exposure of all the cell surfaces to the action of the proteolytic enzyme, the heart ventricles were dissociated in the usual manner with trypsin-EDTA, and then the cells were incubated in fresh trypsin-EDTA solution at 37°C for 30 min. This extended treatment also failed to remove the LSM from the cell surface.

DNase is routinely used before dissociation of trypsin-treated tissues in order to remove deoxyribonucleoprotein (DNP) released from injured cells, and thereby eliminate the clumping of the cells in the DNP gel (33). In the present experiments, the tissue fragments were very sticky while in the trypsin, but lost this stickiness when the trypsin solution was replaced by DNase. All the cells in these experiments were treated with DNase after the trypsin or pronase digestion; therefore, as can be seen in Fig. 2, DNase treatment following proteolytic treatment is also ineffective in removing the LSM. In one experiment control pieces of heart ventricle, without previous incubation in solutions of proteolytic enzymes, were exposed to DNase (0.1 mg/ml, pH 7.3) for 15 min before fixation; these tissue fragments showed the usual LSM at their surfaces.

Trypsin-dissociated cells were treated in different experiments with three mucolytic agents: *N*-acetyl-L-cysteine, α-amylase, and neuraminidase.

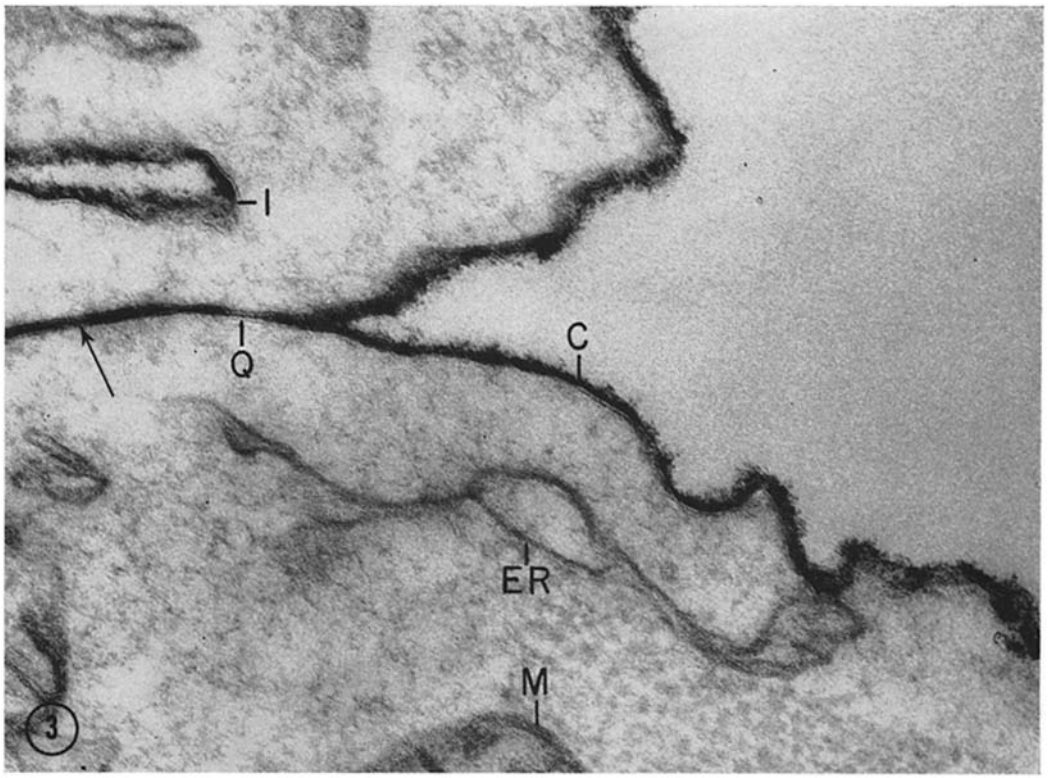

FIGURE 3 Heart ventricle cells, dissociated from the tissue by trypsin-EDTA and treated with 0.5% *N*-acetyl-L-cysteine before fixation. This mucolytic agent has not removed the LSM at the cell surface, which can be seen freely exposed to the medium (*C*), at a junction between the two cells (arrow), and at what is interpreted to be a cross-section through an infolding of the surface into one of the cells (*I*). Mitochondrial membranes (*M*) and endoplasmic reticulum (*ER*) do not have any LSM. One quintuple-layered structure (*Q*) is present in this micrograph. Again, the outer dark leaflet of the cell membrane is indistinguishable from the LSM. × 84,000.

None of these agents, under the conditions used, succeeded in removing the LSM (Fig. 3).

Phospholipase C, at concentrations from 0.001 mg/ml to 5 mg/ml, is capable of stripping the LSM from trypsin-dissociated cells (Fig. 4). At the higher concentrations all of the cells are lysed, but at the lower concentrations (0.01 and 0.001 mg/ml) very few, if any, cells are broken by this procedure. Trypsin-dissociated cells incubated for 20 min in PHL-C at a concentration of 0.001 mg/ml do not have the LSM (Fig. 4), but incubation for 10 min at this concentration is not sufficient to remove the LSM completely from the cells. In the latter case, and also in one experiment in which the cells were treated with heated PHL-C for 20 min, some lanthanum-stained material can be found in a rounded, droplet form either attached to the plasma membrane or free in the space external to the cell (Fig. 5). The diameter of these droplets varies from 300 to 550 A. The plasma membrane of cells denuded of their LSM by PHL-C action has dimensions slightly larger than those of the plasma membrane of trypsin-dissociated cells or of cells in intact tissue fixed in $KMnO_4$. Both dark layers and the light layer are each about 5 A wider in the PHL-C treated cells, making the whole membrane about 15 A wider than normal.

At pH 7.6, solutions of PHL-C can be heated for 10 min at 100°C and still retain 45% of their activity (23, see also reference 27). Because of this heat stability of PHL-C a test of whether removal of the LSM is due to the phospholipase C or some other enzyme contaminating the preparation is to

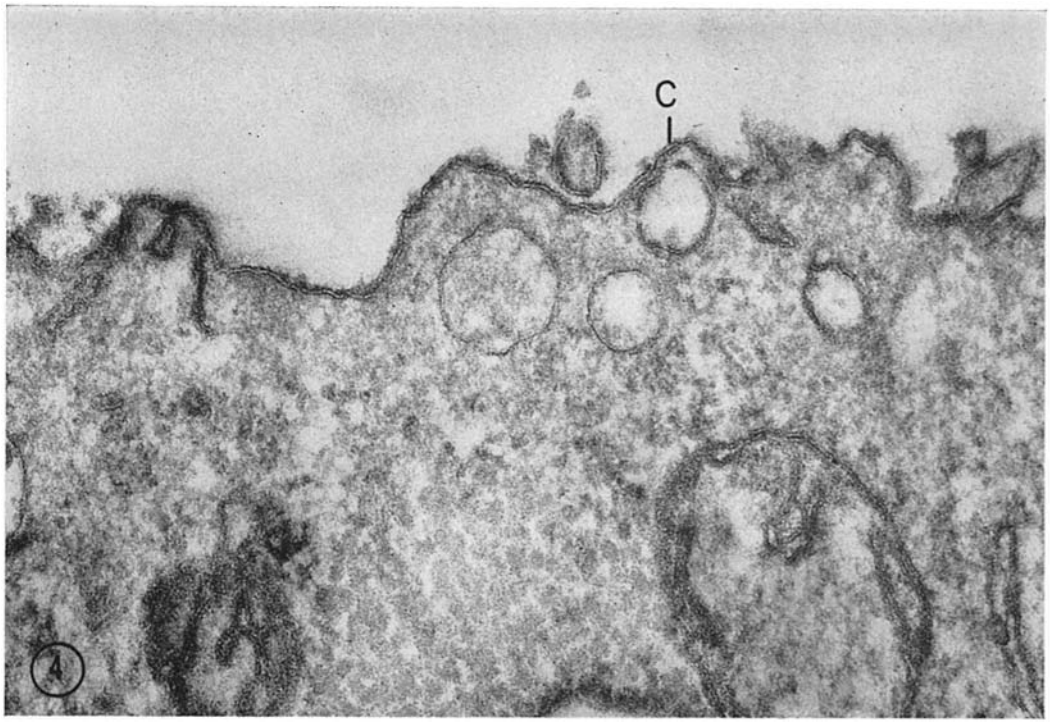

FIGURE 4 Trypsin–EDTA-dissociated heart cell, treated with 0.001 mg/ml PHL-C for 20 min. The LSM has been almost completely removed from the cell membrane (*C*). × 83,000.

heat a solution of the enzyme for 10 min at 60°C. Trypsin-dissociated cells incubated at 37°C for 20 min in heated PHL-C (0.01 mg/ml) are stripped of the layer of LSM external to the cell membrane; in one experiment (Fig. 6) this stripping was more complete than in another one (Fig. 5).

According to MacFarlane and Knight (23, and see reference 16), the activity of PHL-C in the absence of calcium ions is only 10% of the enzyme's activity in the presence of 2.4×10^{-3} M Ca^{++} (see reference 1 for evidence that the required divalent cation is Zn^{++} rather than Ca^{++}). Therefore, another test of whether removal of the LSM is due to the PHL-C or some other component in the preparation is to incubate the cells in a solution of phospholipase C containing EDTA. Trypsin-dissociated cells incubated at 37°C for 10 min in PHL-C plus 0.12% EDTA still have the surface layer of LSM if the concentration of enzyme is 0.01 mg/ml (Fig. 7). When the enzyme is at a concentration of 0.1 mg/ml in the presence of 0.12% EDTA, however, the LSM is partially removed from the cells.

In order to be sure that the LSM was actually being removed by the PHL-C preparation (and was not simply absent or already removed by the dissociation procedure), on each occasion when PHL-C-treated cells were fixed, some trypsin-dissociated cells were also fixed without exposure to the PHL-C. All these trypsin-dissociated control cells had the usual LSM.

It is clear from the experiments in which EDTA was certainly present in excess of any divalent cations added to the solution by the enzyme preparations that this chelating agent also fails to remove the LSM. Such an experiment is the one in which dissociated cells were incubated in 0.01 mg/ml PHL-C plus 0.12% EDTA (Fig. 7).

DISCUSSION

Many different types of cells have previously been found to have one or more layers external to the cell membrane, but these layers were usually found either on single, free-living cells like the ameba (4), on the exposed, luminal surface of such tissue cells as intestinal (17) or kidney (31) cells,

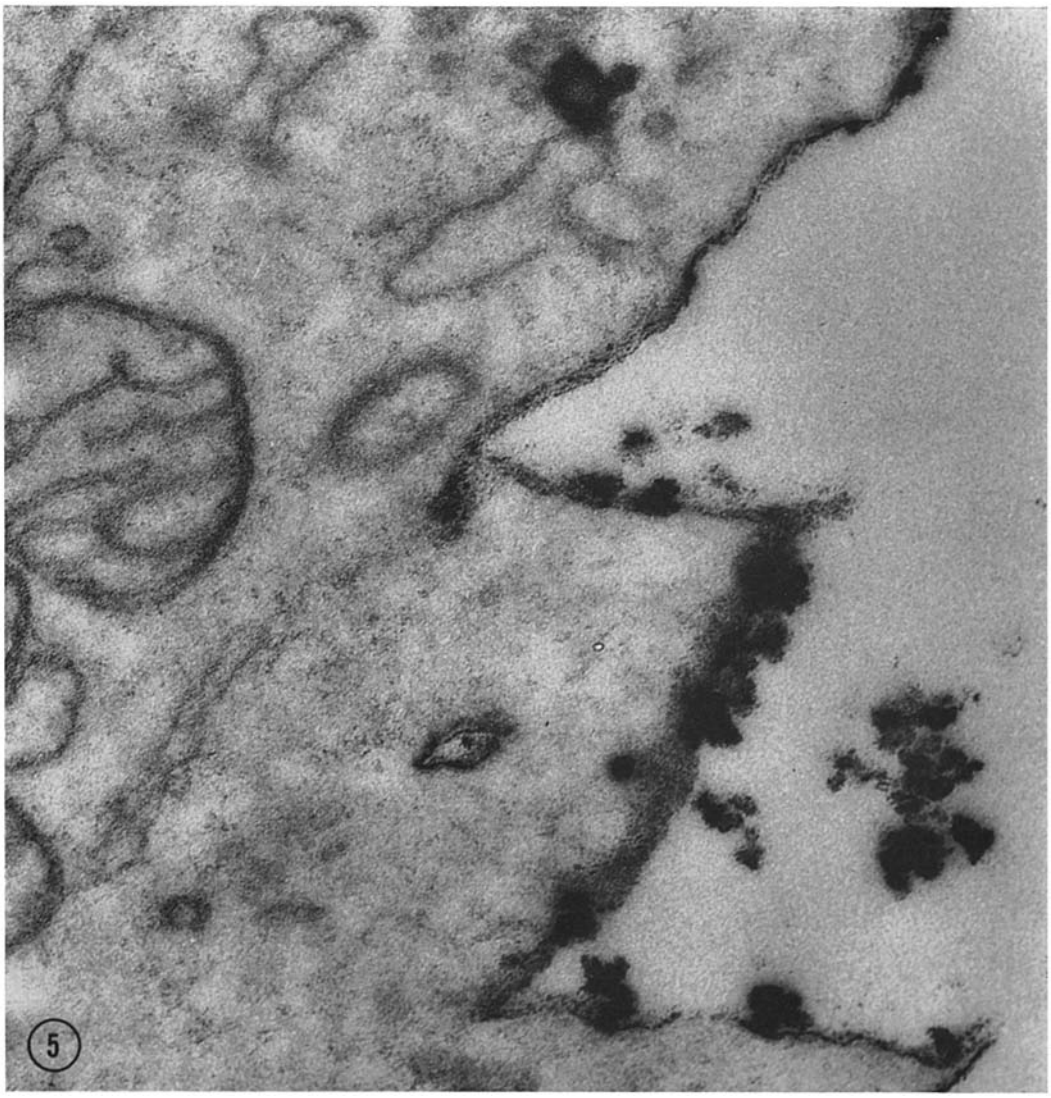

FIGURE 5 Trypsin–EDTA-dissociated heart cell, treated for 20 min with 0.01 mg/ml PHL-C that had been heated at 60°C for 10 min. This cell and the one pictured in Fig. 6 were from two separate experiments. The LSM had not yet been completely removed. Darkly stained material can be seen in various degrees of association with the plasma membrane. × 83,000.

or, if between two cells, at specialized sites such as desmosomes (19). Furthermore, such layers external to the cell membrane have been described mostly in differentiated tissues. Now, with the use of the lanthanum technique, a layer external to the cell membrane can be routinely demonstrated to be present in undifferentiated embryonic cells, even along the junction between cells.

If the LSM is an integral part of the cell, then the contact between cells is an extremely close one; molecules in these intimately apposed cell surfaces can interact, form chemical bonds, and thereby bring about cell-to-cell adhesion. The same sort of chemical bonding may be operative if the LSM is extracellular, but in this case the intermediary of extracellular material would also be involved in cellular adhesion. The LSM is not a part of the cell membrane (understood as the triple-layered

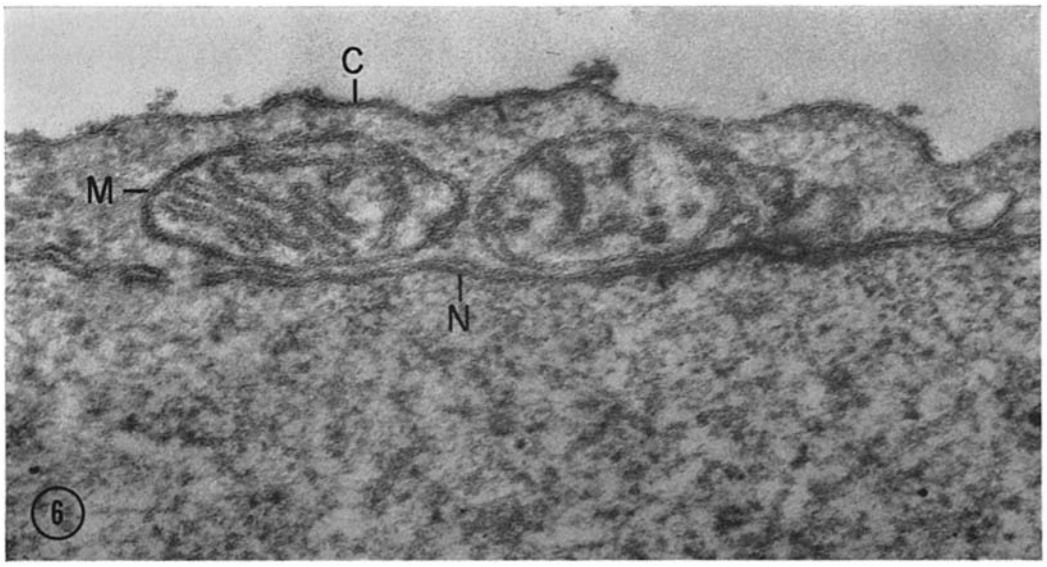

FIGURE 6 Trypsin–EDTA-dissociated heart cell, treated for 20 min with 0.01 mg/ml PHL-C that had been heated at 60°C for 10 min. The LSM has been almost completely removed from the cell membrane (*C*). *M*, mitochondrial membrane; *N*, nuclear membranes. × 83,000.

structure seen in electron micrographs, as mentioned in the Introduction), since the cell membrane remains intact after the LSM has been stripped off by PHL-C treatment. Some protein involved in binding the cells together is probably removed from the region of the LSM by the trypsin or pronase used to dissociate the tissues, even though this cannot be detected by the present lanthanum method. But since much of the LSM remains after dissociation and is stable under all treatments except PHL-C, it seems best to regard the entire LSM, including any part that may be removed by the dissociation procedures, not as some extracellular material but as an integral part of the cell, external to and firmly connected with the cell membrane. Similar reasoning led Ito (17) to conclude that the enteric surface coat on cat intestinal microvilli is probably an integral part of the plasma membrane. This enteric surface coat is a layer of fine filaments whose density is enhanced by double staining with uranyl acetate and lead citrate. In Ito's material, as in ours, the external layer is resistant to EDTA, *N*-acetyl-cysteine, diastase, neuraminidase, trypsin, and pronase; Ito also treated his material with chymotrypsin, hyaluronidase, lysozyme, and papain, and had the same negative result with all these agents (17).

The chemical composition of the LSM remains in question. It may contain lipid, protein, and polysaccharide, either separately or in any of the various possible combinations. The fact that the LSM is removable by treatment with phospholipase C suggests that this outermost layer contains lipid which has been the target of the enzymatic activity. However, as will be discussed later, this is not the only possible mechanism for the removal of the LSM. There are some reports that lipid is present in material external to the cell membrane. For example, Kelly (19) has presented evidence supporting the earlier proposal of Weiss and Ferris (37) that lipid is present in the globular bodies just beneath the basal cells in newt epidermis. Doggenweiler and Frenk (11) report experiments by Rojas in which La^{+++} displaces Ca^{++} from a monolayer of phosphatidylserine or phosphatidylethanolamine, whereas both ions are rejected from a monolayer of phosphatidylcholine. Since there are several different lipid substrates of PHL-C (see reference 1 and references cited therein; 38), and since there is still doubt about the conditions under which some of these substrates can be attacked by PHL-C, no firm conclusions can be drawn about which lipids may be present in the LSM or, alternatively, which lipids are responsible for binding the LSM to the cell membrane.

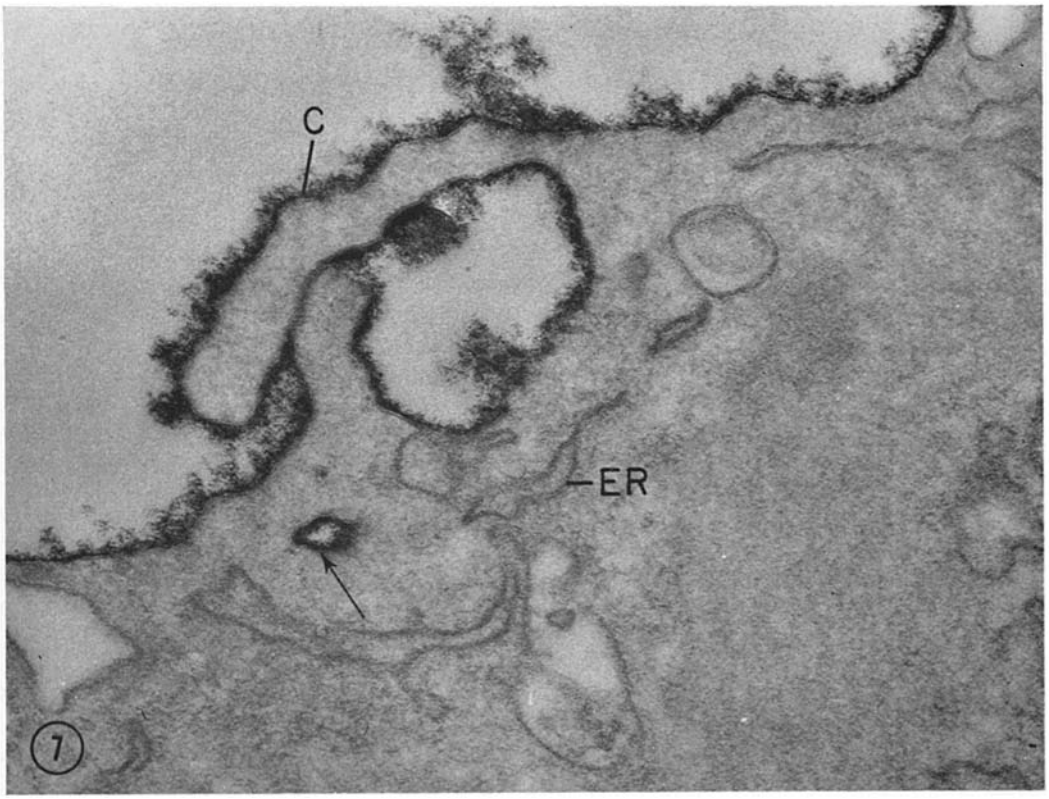

FIGURE 7 Trypsin–EDTA-dissociated heart cell, treated for 10 min with 0.01 mg/ml PHL-C in CMFT with 0.12% EDTA. The LSM has not been removed from the cell surface (*C*). The layer of LSM at the arrow is probably in an infolding of the surface which has been sectioned transversely. *ER*, endoplasmic reticulum. × 84,000.

Another possible constituent of the LSM is protein. That lanthanum ions may bind to protein under certain conditions is indicated by the studies of Srivastava (32). The amount of protein that may be present in the LSM is probably small since trypsin and pronase action produced no effect detectable in the electron micrographs. Some protein, however, must be present in the region of the LSM since these enzymes and collagenase are used to dissociate tissues into a suspension of single cells (7, 15, 25).

Finally, it is possible that the LSM contains polysaccharide. Mathews (24) has reported experiments on the binding of trivalent cations to acid mucopolysaccharides such as chondroitin sulfate and sodium hyaluronate. Bungenberg de Jong (6) states that cations including La^{+++} bind by salt linkage to the ionized carboxyl groups of polysaccharides. Histochemical methods have often shown the presence of acid mucopolysaccharides at the surface of a wide variety of cell types (3, 17, 30), and sialic acid is known to be present in the cell membrane (12). After partial hepatectomy in rats, neuraminidase reduces the electrophoretic mobility of the liver cells and releases *N*-acetylneuraminic acid from the cells (8). Similarly, α-amylase reduces the electrophoretic mobility of rat erythrocytes and releases sialic acid in a bound form (13). This suggests that carbohydrate exists at the outermost surface of these cells. No effect of these two enzymes is noticed in our experiments. Nor could Ito (17) detect any effect of these two enzymes on the enteric surface coat, which nevertheless gave a positive reaction for acid mucopolysaccharide when stained by several different methods for both light and electron microscopy. Perhaps no effect of these two enzymes can be detected by electron microscopic methods be-

cause there may be at the cell surface only a small amount of carbohydrate which can be digested by these enzymes.

The possibility that the PHL-C activity has not removed the LSM, but only rendered it unstainable, is not thought to be very likely. Fig. 5 indicates that some material which is still stainable by lanthanum has been removed from the cells.

Although it is possible that the PHL-C removes the LSM by catalyzing the hydrolysis of lipid components in it, another, and perhaps more probable, mechanism of action would be the splitting of phosphate bonds at or near the junction between the LSM and the outer leaflet of the cell membrane. This would lead to the releasing of chemical groups responsible for the binding of the LSM to the plasma membrane. The enzyme would act, in this case, like a pair of scissors cutting off the LSM from the membrane. When the LSM is freed, it may then fragment and form the small balls seen in our electron micrographs.

There are four indications that the PHL-C and not some contaminant of the only partially purified extract is responsible for the removal of the LSM. First, a very low concentration (0.001 mg/ml) of the preparation succeeds in removing the LSM. Second, some of the expected contaminants such as proteolytic and mucolytic enzymes do not remove the LSM even when these enzymes are used at higher concentrations. Third, heating does not destroy the activity of the preparation. Fourth, removal of divalent cations does significantly lower the ability of the preparation to remove the LSM. Further experiments are planned to test the possibility that some agent other than the PHL-C is removing the LSM.

The possibility that LSM is an artifact must be seriously considered. Its presence at the surface of cells in control tissues, untreated by proteolytic enzymes and other agents, is good evidence that it is not a substance released from the cells during the dissociation process. It is known that DNP is released from cells during dissociation (33) and perhaps also during the cutting of tissues into small fragments. But it is unlikely that this DNP is forming the LSM, since DNase fails to remove the LSM from control tissue pieces and from dissociated cells but does destroy the stickiness of the tissues and cells. The fact that the LSM is tightly bound to the cell and, up to this time, has been found to be released only by a single enzyme, also supports the conclusion that it is not an artifact. Further, the LSM has been found on the surfaces of cells of several types and from different animals, in at least three laboratories in which four different variations of the lanthanum technique have been used. Finally, an entirely different technique has also presented good evidence for the existence of material external to certain regions of the cell membrane and rather firmly attached to it. Benedetti and Emmelot (2), using a negative staining technique on plasma membranes isolated from rat liver, have found small globular units, averaging 50–60 A in diameter, scattered over some areas of the plasma membrane. The width of the LSM is in very good agreement with the diameter of these globules.

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