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SUMMARY The surface of neonatal rat cells in culture, neonatal rat hearts, and adult rabbit hearts have qualitatively similar responses to lanthanum, ruthenium red, and colloidal iron stains. All demonstrate a surface coat and external lamina with abundant negatively charged sites. Cells with intact surface structure do not permit entry of lanthanum (La\(^{3+}\)) intracellularly. The surface of all the myocardial cells studied contained abundant sialic acid distributed in two distinct layers, one in the surface coat next to the lipid bilayer, the other in the external lamina at the interstitial interface. The removal of sialic acid from the cellular surface increases calcium (Ca\(^{2+}\)) exchangeability 5- to 6-fold. Its removal also permits La\(^{3+}\) to enter the cell and displace more than 80% of cellular Ca\(^{2+}\). Despite these marked alterations in Ca\(^{2+}\) and La\(^{3+}\) permeability, sialic acid removal has no effect on potassium (K\(^{+}\)) permeability. This indicates that the integrity of surface coat is critical in the regulation of Ca\(^{2+}\) (and La\(^{3+}\)) exchange but that K\(^{+}\) permeability is controlled at the bilayer region. Exposure of the cells to [Ca\(^{2+}\)]\(_{0}\) = 5 \(\mu\)M produces a change in the surface. A peeling of the external lamina from the surface coat occurs with separation of the two sialic acid layers and the formation of a fluid-filled bleb between them. We propose that Ca\(^{2+}\) removal ruptures Ca\(^{2+}\) carbohydrate couplings (e.g., fucose-Ca-fucose bridges) which may anchor the external lamina to the surface coat. The effect of Ca\(^{2+}\) removal on ionic permeability is not specific. The cell demonstrates increased permeability to both La\(^{3+}\) and K\(^{+}\). This indicates that Ca\(^{2+}\) depletion affects both the surface structure and the bilayer region.

IN 1963, BENNETT\(^{1}\) suggested that acidic mucopolysaccharides are heterogeneous, complex structures.\(^5\)\(^-\)\(^7\) Despite this complexity, there are quite specific probes available for its analysis given the appropriate combination of myocardial tissue and technique. Since it is necessary to use cultured cells to study the effects on ionic exchange of certain probes applied to the surface coat-external lamina, we have shown how alterations in the cell surface produce a pronounced effect on the ultrastructure of the membrane and on the membrane’s permeability to cations. We studied two perturbations: (1) removal of sialic acid residues by purified neuraminidase and (2) removal of Ca\(^{2+}\) by perfusion with Ca-deficient solution.

We began our dissection of surface coat structure and function in the heart with sialic acid because, in many tissues, approximately two-thirds of sialomucin is located at the cell surface external to the lipoprotein portion of the membrane and it is largely responsible for the fixed negative charge found at the surface of a large variety of cells;\(^5\) in addition, sialic acid has been shown to bind Ca\(^{2+}\).\(^{10,11}\) The effect of Ca-deficient perfusion was studied because, although it is not nearly so specific an intervention as sialic acid removal, it produces a very specific and reproducible alteration in surface coat structure\(^2\) with marked changes in membrane permeability.\(^{13,14}\)

Methods

Since one of the most important aspects of this study was the correlation of structure with functional param-
SOLUTIONS AND ENZYMES

The standard perfusate for both kinetic and ultrastructural studies had the following composition (mM): NaCl, 133; KCl, 3.6; CaCl₂, 1.0; MgCl₂, 0.3; glucose, 16.0; N-2-hydroxyethylpiperazin-N'-2-ethanesulfonic acid (HEPES) buffer, 3.0 (pH 7.1). For isotopic studies, ⁴⁰Ca (ICN Corp.) was added at 1.0-1.5 μCi/ml and ⁴²K (New England Nuclear), at 1.0 μCi/ml. Ca-deficient solutions were the same as standard but the Ca²⁺ concentration was lower than 12 μM and, in most cases, less than 5 μM, as measured on an Atomic Absorption Spectrophotometer (Varian Instruments). Highly purified neuraminidase from *Clostridium perfringens* (Worthington Biochemicals) at a concentration of 0.25 U/ml, pH 7.1, was used in both morphological and kinetic studies. This enzyme was purified chromatographically according to the method of Hatton and Regoecci.

To ensure that our enzyme preparation was free of proteolytic and phospholipase activity, the following procedures were followed:
1. ¹²⁵I-labeled albumin (New England Nuclear) was used as substrate for the enzyme at the concentration applied to the cells. No proteolytic activity was detectable.
2. ³²P-Phosphatidylcholine was used as a substrate for the neuraminidase. The extent of hydrolysis was compared with that obtained with phospholipase C. Our neuraminidase preparation had the equivalent of 0.0025 U/ml phospholipase activity. This amount of phospholipase was incubated with the cells and produced no effect on beating or ⁴⁰Ca exchangeability.

ULTRASTRUCTURAL TECHNIQUES

Tissue Preparation

Tissue culture cells grown from neonatal rat hearts, papillary muscles from 6- and 7-day-old neonatal rat hearts, and the interventricular septum from rabbits all were exposed to lanthanum, colloidal iron hydroxide, and ruthenium red, and the pattern of staining was noted in the three preparations.

Tissue culture cells were grown and processed for ultrastructural studies as previously described. All solutions involved in processing the cells for electron microscopy were poured directly onto the cells in the Petri dish. The primary fixative was glutaraldehyde (2%) buffered with 0.1 M cacodylate buffer (pH 7.1). Postfixation in 1% osmium tetroxide was preceded by a brief rinse in 0.1 M cacodylate buffer. The cells were dehydrated in ethanol and then embedded in Epon 812. Gelatin capsules filled with Epon were inverted over the cells in the Petri dish. After curing, the capsules were broken off and cut with a diamond knife on a Porter-Blum MT-2 ultramicrotome.

Sections from tissues exposed to lanthanum, ruthenium red, or colloidal iron were, for the most part, not counterstained. Interventricular septa from the adult rat heart were fixed with buffered glutaraldehyde perfused through the septal artery. This technique for the perfusion fixation of the rabbit septum has been described. Postfixation with osmium tetroxide, dehydration, and embedding were the same as described for the tissue culture cells. The papillary muscles from the neonatal hearts were treated in the same manner.

Colloidal Iron Hydroxide (CIH) Labeling

CIH was chosen as a histochemical probe because of its known specificity for sialic acid. CIH was prepared according to a modification of the procedures of Gasic et al. Fifty milliliters of 0.5 mM FeCl₃ (Fisher Chemicals) were added to a rapidly boiling distilled water (60 ml). After cooling, 10 ml of glacial acetic acid were added to the CIH solution and the pH was adjusted to below 1.8. The tissue culture cells and adult cells were fixed in 2% glutaraldehyde and then rinsed for 30 seconds in 12% acetic acid. The tissue culture preparation was exposed for 40 minutes to CIH while adult heart cells had a 6.5-hr exposure. All cells were washed for 10 minutes with several changes of 12% acetic acid and then postfixed and embedded.

Lanthanum (La) Staining

La³⁺ was chosen as one of the histochemical stains because, aside from being a well known cell surface marker, it has well studied effects on muscle function. In addition, La³⁺ was used as an ultrastructural marker for changes in membrane permeability in the sialic acid and “zero” Ca²⁺ studies.

LaCl₃ (American Chemicals), in concentrations between 1 and 5 mM, was added to the standard perfusate buffered with 3 mM HEPES (pH 7.1). The tissue culture and adult cells were kept in La solution for times varying between 20 minutes and 2 hours, after which the cells were fixed and processed for microscopy. La was not present in any of the fixatives.

Ruthenium Red (RR)

RR was chosen because it is a well known mucopolysaccharide stain, the histochemistry and effects on function of which have been studied in a number of tissues, including muscle. Staining with RR was performed according to the method of Luft. RR (Sigma) was added to both glutaraldehyde and osmium fixatives whereas, in some experiments, RR was present only in the osmium fixative. RR was added to the fixative in concentrations of 1500 ppm from a stock solution made up in distilled H₂O.

TECHNIQUE FOR MEASUREMENT OF ISOTOPIC EXCHANGE

⁴⁰Ca and ⁴²K exchange was monitored by the scintillation-disk flow cell technique. This technique has been modified substantially since it was originally described. It depends on the growth of a cellular monolayer on one surface of each of two disks composed of polystyrene combined with scintillator material (Nuclear Enterprises). The disks are 45 mm in diameter and 1 mm thick and are designed to form a portion of each side of the flow cell chamber illustrated in Figure 1.
The flow cell. Myocardial cells are grown in monolayers on one surface of each of the scintillator disks. The surface with cells attached forms the inner surface of the flow cell wall. The disks are held in place by washers which are screwed down on the periphery of the disks and form a leak-proof seal. The flow cell is inserted into the light-proof well of the spectrometer so that the scintillator disks are less than 4.5 mm from each of two opposed photomultiplier tubes. Perfusion enters the flow cell through the four ports indicated and exits through a single port at the top of the cell. The cells are cultured from the hearts of 2- to 4-day-old neonatal rats following a standard trypsin digestion technique. Modifications of Blondel et al. were used to ensure a high percentage of myoblasts. The myoblasts are poured into a 50-mm culture dish, the bottom of which is essentially covered by the 45-mm scintillator disk. The scintillator disks are pretreated by Falcon Plastics (Oxnard) to increase cellular adhesivity and sterilized by exposure to ultraviolet light prior to culture. After 3 days of culture, the cells are 80-90% myoblastic as defined by electron microscopic examination, are confluent, and beating synchronously.

After 3 days, the disks are removed from the culture medium and mounted in the flow cell with the surfaces to which the cells are attached directed inward in contact with the perfusate flowing through the flow cell. The flow cell then is inserted into the well of a modified Beta-Mate II spectrometer (Beckman Instruments). The well of the spectrometer contains a Lucite jacket which surrounds the flow cell. The purpose of the jacket is to contain inadvertent leaks. Any leak triggers a circuit that sets off an alarm that permits correction of the problem before damage to the spectrometer occurs. Opposing photomultiplier tubes are mounted flush with the Lucite jacket at each side of the flow cell at a distance of 4.5 mm from the outside surface of the scintillator disks in the wall of the flow cell. With the flow cell in place, perfusate passes through four parts and is directed over the surface of the cells attached to the inner surface of the scintillator disks. Effluent exits through a single port at the top of the flow cell. The components of the standard perfusate have been given above (flow rate on uptake, 10 ml/min; on washout, 24 ml/min; chamber volume, 5 ml; temperature, 24°C). The disks, with cells attached, are equilibrated to the nonisotopic perfusion solution for 30 minutes prior to isotopic labeling. ⁴⁵Ca labeling can be followed continuously, since the efficiency for counting the 0.25 MeV β emission from ⁴⁵Ca bound to the cellular layer on the disks is 73%, compared with approximately 5% for ⁴⁵Ca in the perfusion solution flowing past the disks. This is due to the high quenching of the weak β emission by the solution. ⁴²K labeling cannot be followed continuously due to its high emission energy which results in a background level from the labeling solution which obscures the cellular ⁴²K activity. Monitoring of the washout of either isotope presents no problem, however.

The details of the determination of counting efficiency, counting of blanks, and the establishment of nonlimiting perfusion rate have been presented previously. The unique aspect of the technique is the growth of the cells directly on the isotopic detector (scintillator plastic disks). This permits continuous counting of ⁴⁵Ca uptake and ⁴⁵Ca and ⁴²K washout of the cellular layer. In many cases, the responses of isotopic exchange under control conditions and the response to an intervention can be evaluated in the same culture.

**Results**

**HISTOCHEMISTRY OF THE SURFACE COAT-EXTERNAL LAMINA COMPLEX**

**Lanthanum**

To use La³⁺ as a probe, it was essential to define its localization under control conditions in the cultured myoblasts and in adult cells. We varied the concentration of La (1 and 5 mM) and the exposure time (20 minutes and 2 hours) to see whether optimum parameters for staining could be determined. In both tissue culture and adult septal preparations an electron dense precipitate was found covering the myocardial membrane complex (Figs. 2 and 3). The cell surfaces of tissue culture and adult cells showed a 300-600 Å layer of stain which included the outermost leaflet of the lipid bilayer plus surface coat and the external lamina. This pattern of staining occurred whether 1 or 5 mM La³⁺ was used. The longer times of exposure at 5 mM La³⁺ in the adult cells produced a denser precipitate in some cells. The staining intensity of the lipid bilayer varied little, while the surface coat-external lamina staining intensity did vary (Fig. 2a and b). This variation in stain intensity was less obvious in tissue culture cells and the staining pattern, when present, was of a more uniform nature. La was found routinely lining...
the pinocytotic vesicles and the transverse tubules. However, La was not found within the cytoplasm of intact tissue even when cells were exposed to this cation for 2 hours.

The surface of fibroblasts in the tissue culture preparation and surfaces of endothelial cells in the myocardial preparation also stained intensely with La (Fig. 2C).

**Ruthenium Red**

The cell surfaces of the myoblasts, neonatal hearts, and adult myocardial cells react strongly with RR. A dense deposit adheres to the outer leaflet of the unit membrane, the surface coat, and external lamina in both tissues (Fig. 4A and B). The thickness of the deposit was very uniform; in the tissue culture it averaged about 500 Å and in the adult, about 600 Å.

**Colloidal Iron Hydroxide**

In tissue culture, neonatal hearts and the adult cells CIH staining showed electron dense particles ranging from 30 to 200 Å with the granules more or less uniformly distributed along the membrane in a definite two-layered pattern. CIH particles were bound in a uniform manner just external to the unit membrane, presumably bound to the sialic acid component of the surface coat. A second layer was found in the outermost region of the external lamina, leaving an area of approximately 300 Å relatively free of stain material (Fig. 5A).

This layered pattern of CIH binding was present in both tissue culture and adult preparations (Fig. 5A and B). However, it was seen less frequently in tissue culture cells where section orientation most often is oblique. In adult cells, CIH stain is found lining the transverse tubules.

**SIALIC ACID**

**Effect of Sialic Acid Removal–Ultrastructure**

In this series of experiments the myoblasts were exposed to purified neuraminidase, 0.25 U/ml, for 15 minutes

![Figure 2a: Portion of a cell from the adult rabbit septum that was exposed for 20 minutes to 1 mM LaCl₃ before fixation. Note intense La staining on entire membrane complex (arrows). Section is not counterstained. 40,000x.](image)

![Figure 3: Myoblast cells grown in culture and exposed for 20 minutes to 1 mM La³⁺ before fixation. Arrow heads point to lanthanum staining of cellular surface. Dense intracellular particles are ribosomes, which are abundant in these developing cells. Section not counterstained. Mfi, myofilaments; Z, Z line. 30,000x.](image)
followed by exposure to either lanthanum, CIH, or RR.

Lanthanum. Figure 6 illustrates a typical myoblast exposed to La$^{3+}$ after removal of sialic acid. La can be seen throughout the cytoplasm, with the greatest disposition around the mitochondria. La staining of the surface is decreased and most of the stain is seen within the cells.

CIH. Incubation of myoblasts with neuraminidase greatly reduced the number of membrane-bound CIH particles. In every tissue culture cell not treated with neuraminidase, CIH staining showed a uniform amount of particles as illustrated in Figure 5B, whereas, after neuraminidase treatment, CIH staining was almost eliminated, as illustrated in Figure 7.

RR. Removal of sialic acid from the cell surface had the least effect on RR staining. In untreated cells, we measured an average thickness of RR precipitate of 500 Å (Fig. 4B) and, after sialic acid removal, RR stain was reduced to an average thickness of 350 Å (Fig. 8).
**Effect of Sialic Acid Removal–Ionic Exchange**

**Calcium.** Figure 9a compares the pattern of ⁴⁵Ca uptake and the response to the addition of 0.5 mM lanthanum in untreated and neuraminidase-treated cultured cells. The "control" and neuraminidase cultures were treated identically except that the curve indicated "neuraminidase" is from cells exposed to the enzyme at a concentration of 0.25 U/ml for 15 minutes immediately prior to exposure to ⁴⁵Ca-labeled perfusate. The counts are normalized for cell mass and isotopic specific activity and are, therefore, directly comparable. Note that, in the control, asymptotic ⁴⁵Ca activity is reached after 40–45 minutes of labeling, which is typical for cardiac cells in monolayer culture. This is in contrast to the cells exposed to neuraminidase in which asymptotic activity is achieved in 7–8 minutes. This pattern, which indicates a 5- to 6-fold increase in the rate of Ca²⁺ exchange, was reproduced in 18 cultures.

The experiments illustrated in Figure 9A suggest a lower value of exchangeable Ca²⁺ after neuraminidase treatment. However, the total series of 24 control and 18 neuraminidase-treated cultures does not confirm this. In the control experiments, the exchangeable Ca²⁺, as indicated by the asymptotic level for ⁴⁵Ca labeling, is 16.2 ± 1.4 (se) mmol/kg dry cells. The neuraminidase-treated cells averaged 18.5 ± 1.4 mmol Ca²⁺/kg dry cells. These total exchangeable contents are not significantly different (P > 0.25).

The effect of addition of 0.5 mM La³⁺ to the ⁴⁵Ca-labeled perfusate after asymptote had been achieved also is shown in Figure 9A; 12.9% of exchangeable Ca²⁺ was displaced from the control cells, whereas 91.5% is displaced from the cells previously exposed to neuraminidase. In a series of 10 control experiments, 0.5 mM La displaced 1.84 ± 0.13 mmol Ca²⁺/kg dry weight or 11.4% of labeled Ca²⁺. In a series of 11 cultures pretreated with neuraminidase (0.25–0.33 U/ml) for 10–15 minutes, La³⁺ displaced 15.6 ± 1.27 mmol Ca²⁺/kg dry weight or 83% of labeled Ca²⁺. This displacement compared with control is different at a high level of significance (P < 0.001).

The effect of neuraminidase treatment on ⁴⁵Ca washout, compared with control, is illustrated in Figure 9B and indicates that 95% of the ⁴⁵Ca label washes out within 5 minutes. This is compared with the control in which 25% of the label washes out within the initial 5-minute period. The washout pattern emphasizes the marked increase in Ca exchangeability induced by pretreatment with the enzyme and was virtually identical in four experiments.

**Potassium.** The effect of sialic acid removal on potassium exchangeability was evaluated by comparison of the pattern of ⁴²K washout from four control cultures and four cultures exposed to neuraminidase (0.25 U/ml) for 15 minutes prior to ⁴²K labeling and washout, exactly as had been done in the ⁴⁵Ca-labeling and washout studies. Representative washouts are illustrated in Figure 10. The mean rate constant for the four control cultures in this series is 0.059 ± 0.004 min⁻¹ (range, 0.051–0.068) and for the four neuraminidase-treated cultures is 0.061 ± 0.008 min⁻¹ (range, 0.036–0.072). The washout patterns are monoexponential in both cases and are not significantly different.

**Sialic Acid Content**

The average sialic acid content of two typical cultures as measured by the method of Warren is 80.8 × 10⁻⁹ mol cellular protein per mg. Exposure of these cells to neuraminidase (0.25 U/ml) for 15 minutes, as was done in the structural and isotopic exchange studies, caused 49.3 × 10⁻⁹ mol protein per mg to be released into the supernatant fluid, or 61% of the total cellular sialic acid.

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**Figure 5 A:** Electron micrograph from adult septum stained with colloidal iron hydroxide (CIH). Note double-layered pattern of the stain. CIH stains outermost region of external lamina (EL) and stains the surface coat (SC) just superficial to lipid bilayer leaving an area of approximately 300 Å free of stain. Mfl, myofilaments; Mit, mitochondria; Z, Z line. 68,000x. B: Electron micrograph of tissue culture cell exposed to colloidal iron stain. External lamina (EL) and surface coat (SC) are stained in a similar pattern to that seen in the adult (Fig. 5A). 60,000x.
content. No significant amounts of free sialic acid were released into the supernatant fluid from cells exposed to control perfusate solution, i.e., without neuraminidase.

**“ZERO” CALCIUM**

Zero [Ca\(^{2+}\)]\(_0\) Perfusion-Ultrastructure

Both preparations were perfused with Ca-deficient perfusate (<5 mM) for 1 hour in tissue culture experiments and for 20 minutes in adult myocardium, followed by 20 minutes of exposure to 1 mM La\(^{3+}\). Under these conditions, La was found to enter the cells, and deposits of stain were seen in the cytoplasm of the tissue culture and adult cells. Figure 11A is an unstained section of a myoblast exposed to zero Ca\(^{2+}\). A peppery precipitate of La can be seen throughout the cytoplasm and within the mitochondria. Figure 11B is an unstained section from zero Ca\(^{2+}\)-perfused septum. The intracellular disposition of La is clearly seen.

Other striking changes in the cell surface were seen after zero Ca\(^{2+}\) perfusion. In a typical cell, the external lamina is peeled or separated from the surface coat in over 60% of the cell perimeter. This is clearly shown in Figure 12 where a zero Ca\(^{2+}\)-treated cell was stained with CIH. The external lamina as it separated from the surface coat formed a bleb (0.5 \(\mu m\) at its maximum dimension) which remained anchored to the membrane at the transverse tubules.

In addition, in both the tissue culture and the adult myocardium, the cell surface became less rectilinear after zero Ca\(^{2+}\) perfusion and had more abundant “villi.” In the case of myoblasts and in the adult cells, many finger-like protrusions were seen indicating an increase in deformity or mobility of the membrane.

**Zero [Ca\(^{2+}\)]\(_0\) Perfusion-Ionic Exchange**

**Calcium.** To evaluate the effect of severe Ca\(^{2+}\) depletion on ionic exchange, cultures were exposed to solutions...
**FIGURE 7** Portions of two myoblasts pretreated with neuraminidase (0.25 U/ml) and then exposed to colloidal iron hydroxide. Note marked decrease in stain reaction on cell surface. Compare with Figure 5B. Mf, myofilaments; ID, intercalated disks. Section is not counterstained. 28,000×.

**FIGURE 8** Electron micrograph of myoblast treated with neuraminidase and then ruthenium red (RR). Intense RR stain is seen on cell surface (arrows). Mt, mitochondria. Section has been lightly counterstained with uranyl acetate. 40,000×.

**FIGURE 9** A: ⁴⁰Ca uptake in control and neuraminidase-treated cultured heart cells. The curve marked “neuraminidase” is from cells treated with the enzyme at a concentration of 0.25 U/ml for 15 minutes immediately prior to exposure to ⁴⁰Ca-labeled perfusate. La³⁺ (0.5 mM) was added to the isotopically labeled solution in both the control and neuraminidase-treated cultures after asymptote had been reached. Note the marked increase in rate of ⁴⁰Ca exchange and the greatly increased displacement of ⁴⁰Ca by La³⁺ in the neuraminidase-treated cells. B: ⁴⁰Ca washout from control and neuraminidase-treated cells. Cells were treated as described in Figure 9A. Washout was instituted after asymptotic labeling had been achieved in each case. Note the greatly increased ⁴⁰Ca washout rate from the enzymatically treated cells consistent with greatly increased Ca²⁺ exchangeability after neuraminidase treatment.
that contained less than 12.5 μM [Ca2+]o for periods of 60–90 minutes. The cells were then labeled with 45Ca to asymptotic levels with perfusate that contained 1.0 mM [Ca2+]o. At asymptote, 0.5 mM La3+ was added to the perfusate and the displacement of Ca2+ was measured. One of four such experiments is illustrated in Figure 13A. The rate of 45Ca uptake is not significantly altered, but the quantity of Ca2+ displaced by La3+ is greatly increased. In the experiment illustrated, 0.5 mM La3+ displaced 4.9 mmol Ca2+/kg dry weight or 48% of the exchangeable Ca2+.

In the four experiments, La3+ displaced 5.2 ± 0.3 mmol Ca2+/kg dry weight, which is significantly (P << 0.001) greater than the displacement in the control series. The average percentage displacement is 40.5%, compared with 11.4% in the controls. Therefore, despite the fact that the cells are exposed to normal [Ca2+]o levels during the course of 45Ca labeling, the cellular Ca2+ accessible to La3+ displacement remains increased subsequent to the exposure to zero [Ca2+]o perfusate.

**Potassium.** The effect of zero [Ca2+]o exposure on potassium exchange is illustrated in Figure 13B. The level of [Ca2+]o in this series was approximately 5 μM. As can be seen from the figure, the rate of 42K loss increases immediately upon Ca-free perfusion. The subsequent exchange is clearly not monoexponential, but an average rate is approximately 0.133 min⁻¹ for the initial 10 minutes following zero [Ca2+]o perfusion. This is an increase of 77% in the rate of 42K loss. Such an increase was found in four cultures with the average rate of 42K loss increasing from 0.059 min⁻¹ to 0.093 min⁻¹ or by 60%.
As shown in Figure 13B, the rate tends to decrease upon continued perfusion with zero \([Ca^{2+}]_0\). This nonlinearity favors a net loss of K\(^+\) rather than the induction of a more rapid steady state exchange by zero \([Ca^{2+}]_0\) perfusion.

**Discussion**

**ULTRASTRUCTURE HISTOCHEMISTRY**

The results indicate an essential similarity among cells cultured from the neonatal rat heart, from the neonatal rat papillary muscle, and from the rabbit heart with respect to surface sites. These results are clearly qualitative but confirm the presence of sites to which lanthanum, ruthenium red, and colloidal iron bind. All three types of cells demonstrate the two types of surface layers common to the periphery of most animal cells: (1) a surface coat, glycoprotein in nature, which is integrated with the plasma membrane, and (2) the more peripheral external lamina composed of protein and carbohydrate and located immediately adjacent to the interstitium and its contents.

**LANTHANUM**

Lanthanum has been successfully used as a stain for cell surfaces and for delineation of the extracellular space.\(^4\)\(^-\)\(^21\) In our work we have used ionic lanthanum as a stain for the myocardial membrane complex. In both cultured myoblasts and myocardial cells from adult rabbits, lanthanum stained the lipid bilayer, the surface coat and the external lamina (Fig. 2a and b, and Fig. 3). In addition, nonmuscle cells (fibroblasts and endothelial cells) in both these preparations were routinely found to be stained with lanthanum (Fig. 2c). Martinez-Palomo et al.,\(^22\) in a study in the adult mammalian myocardium, found selective binding of ionic lanthanum solely on cardiac cells. The authors suggested that the specificity they observed could be the result of selective wash-off from noncardiac membranes, indicating, however, a high affinity of lanthanum for cardiac cell membranes. Our results seem to indicate that lanthanum binds to the myocardial membrane complex with different affinities, since we found the staining intensity of the lipid bilayer very reproducible while the staining of the surface coat-external lamina varied from light to heavy deposits of stain (Fig. 2). This variation is probably the result of a wash-off of lanthanum from these sites and may well indicate a higher affinity of lanthanum for the lipid bilayer. It is interesting that Lesseps\(^30\) found that only phospholipase C treatment would totally remove the lanthanum staining material on the cell surface on a variety of tissues indicating La\(^{3+}\) affinity for lipid moieties within the membrane.

While the intensity of lanthanum staining may vary, there is agreement from numerous studies on different
tissues that lanthanum stains cell surfaces and is not found intracellularly in intact tissue. Our results clearly show that lanthanum is restricted to the cell surface by a normal intact membrane complex, because when perturbations are introduced to the surface coat-external lamina, e.g., removing sialic acid molecules or calcium ions, lanthanum is found intracellularly (Figs. 6, 11A and B). The localization of lanthanum to surface membrane sites becomes extremely important since we know lanthanum binding in tissue culture cells functionally displaces 10-20% total exchangeable calcium, while eliminating further calcium exchange;4 in the adult myocardium, lanthanum binding results in uncoupling of excitation from contraction.5

**RUTHERNUIUM RED**

RR stains more than sialic acid molecules and is considered a general polyamionic stain. Our results showed that, after neuraminidase treatment, there remained significant RR on the cell surface (Fig. 8). This is in agreement with the findings of Luft who noted little precipitation when RR reacted with sialic acid. Morgan, working with fibroblasts, and Huet and Herzberg, working with hamster embryo cells, noticed only a slight decrease in RR staining after neuraminidase treatment.

**COLOIDAL IRON HYDROXIDE**

CIH is bound to a variety of cell membranes by coulombic interaction with negatively charged surface groups, predominantly from sialic acid. The stain is applied at pH 1.8 where most anionic surface groups are uncharged, whereas sialic acid with a pKₐ 2.7 has approximately 20% of its carboxyl groups ionized. The specificity of CIH for sialic acid has been demonstrated by Nicolson in red blood cells where neuraminidase reduced membrane-bound sialic acid by more than 90% and concurrently reduced CIH labeling to below 15% of untreated controls. Benedetti and Emmelot also demonstrated CIH specificity for sialic acid in liver cell membranes.

The interesting pattern of CIH staining, consisting of one layer of CIH particles on the surface coat and the other on the outermost region of the external lamina with an approximately 300 Å zone free of particles, was present in both adult and neonatal hearts and tissue culture cells (Fig. 5A and B). This indicates that sialic acid molecules with their negatively charged carboxyl groups are present next to the lipid bilayer and on the external lamina at the interstitial interface. This unique pattern of CIH staining and its indication of sialic acid localization has been recognized in skeletal muscle by Zachs et al. and, from the micrographs by Howse et al. in their study on myocardial cell surface, appears to be present in crayfish and sheep hearts.

**SIALIC ACID REMOVAL**

Exposure of cultured cells to purified neuraminidase (0.25 U/ml) resulted in the release of 61% of the total cellular sialic acid. Though longer exposure at higher concentrations of enzyme may have augmented the release, Winzler noted that, in many cells, the maximum release is 50-70% of total sialic acid. It is clear from the decrease in CIH staining (Fig. 7) that a major fraction of the sialic acid is removed from the surface coat-external lamina complex. The lack of CIH staining and some intracellular swelling were the only structural changes noted for the myoblasts after sialic acid removal.

The effect of this removal upon Ca²⁺ exchange is striking. The rate of ⁴⁰Ca uptake and washout is increased 5- to 6-fold and the La ion, normally restricted to the lipid bilayer, enters the cell (Fig. 6) and displaces more than 80% of the exchangeable Ca²⁺. The effect of sialic acid removal on Ca²⁺ exchange is in marked contrast to its effect on potassium exchange (Fig. 10). K⁺ exchangeability was not significantly affected. This lack of effect on K⁺ exchange in heart cells is in essential agreement with the effects reported in leukemic cells and in Erhlich ascites cells where a decreased exchangeability actually was found. Therefore, sialic acid removal does not result in a nonspecific disruption of cellular ionic permeability nor does it
produce cell death. Cultures were exposed to the enzyme in the standard manner, followed by extensive washing in normal medium, and the cells were reincubated. After 48 hours, the cells remained retracted on the culture dish and Ca\(^{2+}\) exchangeability was still high but many of the cells were beating asynchronously.

The exchange studies indicate that, though the surface coats are changed, the lipid bilayer remains intact. Studies in nerve and in heart muscle utilizing tetraethylammonium ion as a blocking agent for K\(^{+}\) conductance\(^{25,26}\) are consistent with the concept that the sites for control of K\(^{+}\) flux are channels through the lipid bilayer. Although it is not possible to rule out subtle changes in the lipoprotein, it is certain that sialic acid removal leaves this component of the membrane essentially intact. The study of Pape et al.\(^{20}\) in which 80% of the sialic acid was removed from Amphiuma red blood cells without any change in transmembrane potential, supports the lack of effect on potassium permeability. The results of the present study also agree with the proposal by Kraemer\(^{14}\) that most of the surface sialic acid could be removed without destroying the "permeability barrier" for potassium. Despite maintenance of this barrier, the movements of Ca\(^{2+}\) into and out of the cell are grossly altered.

Sialic acid is always the terminal group on the saccharide side chains of the glycoproteins. It is this terminal location and the fact that it is negatively charged at physiological pH which contributes to its major role in the determination of the characteristics of membrane glycoproteins. The negative charge of the molecule makes it an excellent candidate for Ca\(^{2+}\) binding. Studies differ, however, as to the magnitude of the binding. Forrester\(^{40}\) found that Ca\(^{2+}\) did not reduce the electrophoretic mobility in red blood cells where the total net negative surface charge is generated by sialic acid residues. On the other hand, Long and Mouat\(^{19}\) found that 65% of the total Ca\(^{2+}\) bound to human red cells could be attributed to sialic acid when the cells were exposed to 2.5 mM Ca\(^{2+}\) in an isotonic sucrose medium. This non-ionic medium would enhance Ca\(^{2+}\) binding. Slutz and Marinetti\(^{11}\) found, in rat liver plasma membrane, that sialic acid accounted for about 30% of the Ca\(^{2+}\) binding, with acidic phospholipids accounting for the remainder. The fact that neuraminidase treatment reduces surface site binding of La\(^{3+}\) (Fig. 6), added to the fact that La\(^{3+}\) competes for and displaces Ca\(^{2+}\) from rapidly exchangeable cellular sites,\(^{4}\) indicates that sialic acid residues account for a significant number of the superficial Ca-binding sites in cardiac tissue.

It seems reasonable to propose, on the basis of the above analysis, that Ca\(^{2+}\) binding to sialic acid residues in the surface coat-external lamina complex plays a crucial role in the regulation of transmembrane Ca\(^{2+}\) flux in the heart. Removal of this nine carbon amino sugar essentially destroys the ability of the cell to regulate its Ca\(^{2+}\) exchange and in addition permits entry into the cell of competitive cations such as La\(^{3+}\) which normally do not pass the sarcolemma. It is clear that an intact lipid bilayer, at least with respect to prevention of potassium leakage, is not sufficient to prevent a marked increase of Ca\(^{2+}\) (and La\(^{3+}\)) permeability. It has been demonstrated by \(^{4}\)Ca kinetic studies in functional ventricular tissue\(^{4}\) that, in the face of increased concentrations of extracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{o}\) to 12 mM), intracellular Ca\(^{2+}\) levels are prevented from rising to a value that would induce contraction. This is not accomplished by an increasingly rapid turnover as [Ca\(^{2+}\)]\(_{o}\) increases, i.e., high influx matched by a high efflux of Ca\(^{2+}\), but by a restriction of influx. It was proposed that this control of influx might be based upon a decrease in membrane permeability "as negative charges in the membrane are neutralized."\(^{1}\) The present study points strongly to sialic acid residues as the locus of the negative charge and/or as critical to the organization of the surface structure which controls access of Ca\(^{2+}\) to the cell. As Lloyd\(^{26}\) states, "Because sialic acid is negatively charged, its removal does not merely lead to the exposure of the subterminal sugar or the loss of negative charge . . . but also to a change in the shape of the sialoglycoprotein, and hence it relationship with nearby molecules, that could equally modify behavior."

**ZERO Ca PERFUSION**

Exposure of heart cells to [Ca\(^{2+}\)]\(_{o}\) concentrations which approach zero induces a specific change in the structure of the surface coat-external lamina complex. Muir\(^{12}\) noted that the basement membrane was separated from the surface of the cell, and this change was preceded by separation of the intercalated disks. The present results show, with the aid of the colloidal iron stain, that zero [Ca\(^{2+}\)]\(_{o}\) does not result in separation of the entire surface complex but separates the external lamina layer from the surface coat. The two regions stained by colloidal iron separate by 0.5 μm (at the point of maxima separation) and a space is formed between them. This results in a bleb that stretches from "T" tube to "T" tube (Fig. 12). The external lamina remains anchored to the cell since its extension into the T system is usually not broken. It appears that Ca-dependent connections between external lamina and surface coat are broken and, subsequently, the two layers are separated by a fluid-filled space.

It is well recognized that the Ca ion is of importance in cell to cell adhesion, but it seems clear that it is also important in the maintenance of connections between the external lamina and surface coat. The basis of these connections may be calcium carbohydrate bridges as described by Cook and Bugg.\(^{43}\) Specifically, they described the situation in which Ca\(^{2+}\) is chelated by a pair of hydroxyl groups for each of two α-fucose molecules. Fucose, as is sialic acid, is a common terminal sugar of oligosaccharides or glycoproteins, and Ca\(^{2+}\) forms the link in hydrated fucose-calcium-fucose bridges. It seems not unreasonable to speculate that Ca\(^{2+}\) removal might result in rupture of these carbohydrate couplings at the external lamina-surface coat interface. Another possibility is that exposure of the cell membrane to a Ca-deficient environment effects the conformations of membranous integral proteins. This possibility is currently under investigation.

As stated, it appears that the surface is deformed into blebs or fluid-filled sacs after Ca\(^{2+}\) removal (Fig. 12). This implies that the blebs are formed by accumulation of fluid in the potential space created by rupture of the linkage between the external lamina and the surface coat. If such is the case, it is likely that osmotically active components
have leaked into this region either from the extracellular space or from the cell. Although the observed structural change following Ca²⁺ depletion is quite specific, the effect on ionic permeabilities is not. It is striking that, in both tissue culture and adult hearts exposed to zero Ca²⁺ perfusion, La³⁺ is found intracellularly and Ca²⁺ is displaced (Fig. 11A and B). In addition, potassium permeability of the cultured cells increases almost immediately upon zero Ca²⁺ perfusion (Fig. 13B). These permeability changes are consistent with the findings of Paradise and Visscher⁴ in rabbit hearts exposed to zero Ca²⁺ for various periods of time and reperfused with Ca-containing solution. Zimmerman and Hülsmann¹⁴ were the first to describe what they termed the “Ca paradox” as a bleaching of the heart upon return to Ca-containing perfusion. This was found to be due to a loss of myoglobin. In addition, other large molecules such as lactic dehydrogenase and creatine phosphokinase were lost, as well as phosphorylase. Visccher and Visscher² found that, attending the marked contracture produced upon reintroduction of Ca²⁺, there was a 30% increase in Ca content of the tissue.

The evidence indicates that, in addition to major changes in the external lamina-surface coat complex, zero [Ca²⁺]₀ perfusion produces defects in the lipid bilayer. As noted previously, the acidic phospholipids, which form the polar hydrophilic components of the lipid bilayer, are major sites of Ca²⁺ binding. Depletion of Ca²⁺ at these sites might be expected to alter structure significantly with the result that potassium channels are opened, as well as defects produced, which permit the passage of larger molecules.

Acknowledgments

We thank Eileen Danhof and Sarah Beydler for their fine assistance with the electron microscopy, and Mira Maruşıch for their fine assistance with the biochemical determinations.

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Circ Res. 1977;41:702-714
doi: 10.1161/01.RES.41.5.702
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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