A ROLE FOR ANIONIC SITES IN EPITHELIAL ARCHITECTURE

Effects of Cationic Polymers on Cell Membrane Structure

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ABSTRACT

The effects of several cationic polymers (poly-L-lysines, protamine, and histone) on rabbit gall bladder epithelial cells were studied to explore possible roles for negative sites in the membrane. The tissue was bathed for 30 min at 37°C in Ringer's solutions containing from 0.1 to 100.0 μg/ml of cationic polymers, and subsequently was fixed with 1% OsO_4 and examined with the electron microscope. All cationic polymers, at appropriate concentrations, produced similar changes in membrane structure. Adjacent membranes frequently were fused. Membrane structures such as microvilli lost rigidity. Cell membranes showed an apparent increase in permeability as judged by osmotically traumatized cells. These results indicate that fixed anionic sites play significant roles in stabilizing epithelial membrane structures.

INTRODUCTION

Fixed negatively charged sites are recognized as a universal characteristic of cell membranes (Cook, 1968; Weiss, 1970). As the subject of much speculation, anionic sites have been proposed for such roles as: (*a*) cell-to-cell adhesion and aggregation (Kemp, 1968; Humphreys, 1965); (*b*) membrane integrity (Glaeser and Mel, 1966; Weiss, 1965); (*c*) cell recognition (Glaeser and Mel, 1966); (*d*) nerve excitability (Singer and Tasaki, 1968); (*e*) cation transport (Glick and Glithens, 1965; Weiss and Levinson, 1969); and (*f*) antigenic response (Springer, 1970). It is suggested that the polyanionic behavior of membrane components contributes buffering capacities when the cell is exposed to pH, osmotic, or mechanical shock (Katchalsky, 1964; Philpott, 1968; Uhlenbruck, 1971). Fixed anions may contribute significantly to ion selectivity and molecular sieving properties of the cell membrane (Eisenman, 1969; Mohos and Skoza, 1969; Diamond and Wright, 1969).

Even though it is easily conceivable that membrane anionic sites may be multifunctional and vary in specific function from cell to cell, the universality of such chemistry suggests that anionic sites may participate in underlying phenomena which are essential to cell function in general.

It is known that poly-L-lysine, a cationic polymer, is adsorbed selectively to the plasma membrane (Mamelak et al., 1969) and that most probably the adsorption is due to an electrostatic interaction with the negative charges of the membrane (Katchalsky et al., 1959). Therefore, we have examined several parameters of the rabbit gall bladder epithelium before and after

exposing the mucosa to various cationic polymers in order to determine (*a*) whether such neutralization might produce changes in membrane morphology and physiology which would indicate elemental functions of fixed anions in membranes, and (*b*) whether the effects of poly-L-lysine adsorption are due to the specific properties of this compound or to nonspecific neutralization of the membrane anions.

This communication reports the morphological effects of exposing the gall bladder mucosa to various concentrations of several distinct cationic polymers, i.e., poly-L-lysine (two molecular weights), protamine, and arginine-rich histone. A subsequent paper will describe the physiological effects of exposing the tissue to poly-L-lysine. The results of the present experiments corroborate previous observations that poly-L-lysine induces membrane-to-membrane adhesion (Katchalsky et al., 1959) and cell leaching (Mamelak et al., 1969), but demonstrate further that the phenomena are the results of cell membrane interactions with cationic polymers in general, and thereby emphasize that membrane-fixed anions play significant roles in stabilizing the membrane as a morphological structure and permeability barrier, and in forming membrane-to-membrane interactions.

MATERIALS AND METHODS

Solutions

Solutions of poly-L-lysine HBr, mol wt 175,000 (lot #78B-0890, Sigma Chemical Co., St. Louis, Mo.) and poly-L-lysine, mol wt 2,800 (lot #59B-5010, Sigma Chemical Co.) were prepared in the following concentrations: (*a*) 0.1 μg/ml, (*b*) 1.0 μg/ml, (*c*) 10.0 μg/ml, and (*d*) 100.0 μg/ml. Solutions of protamine sulfate (lot #3-17952, K & K Laboratories, Inc., Hollywood, Calif.) and arginine-rich histone (lot #118B-1480, Sigma Chemical Co.) were made at all but the lowest of the above concentrations. All polymeric cations were dissolved to the appropriate concentration in a gall bladder Ringer's solution containing millimolal concentrations of the following solutes: 110.0 mM NaCl, 7.0 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11.1 mM glucose, 1.2 mM NaH_2PO_4, and 25.0 mM $NaHCO_3$ (Machen and Diamond, 1969). The anionic polymer, heparin (lot #18B-0800, Sigma Chemical Co.), was dissolved in Ringer's solution to a concentration of 200.0 μg/ml. (The purity and molecular weight of the compounds as given by the respective suppliers were verified in this laboratory by synthetic boundary ultracentrifugation [Schachman, 1959], Sephadex gel filtration [Fischer, 1971], and disk electrophoresis [Davis, 1964].)

Experimental

New Zealand white rabbits (2–3 kg weight) were anesthetized with 3–4 ml of 5% sodium pentabarbital administered intravenously. The gall bladders were removed immediately, rinsed with cold Ringer's solution, everted, and thoroughly rinsed again with cold Ringer's solution. Small strips (ca. 2 × 5 ml) were cut from the bladder and carefully mounted, mucosa facing outward, on strips of dental wax. The tissue strips were then bathed for 30 min in solutions containing the appropriate concentration of the respective cationic polymer or test compound. All solutions were maintained at 37°C and continuously stirred by bubbling 5% CO_2-95% O_2 through the bath. Controls for all experiments consisted of bathing tissue strips mounted on dental wax for a 30 min period in Ringer's solution only.

Microscopy

After incubation in the appropriate bathing solution, the tissue was fixed for 1 h at 4°C, with 1% OsO_4 dissolved in a 0.15 M sodium phosphate buffer at pH 7.3. Conventional techniques of dehydration and embedding were employed (Luft, 1961). Sections were cut to 500–700 A thickness and double stained with uranyl acetate and lead citrate solutions (Reynolds, 1963). Sections were examined with either the RCA EMU-3F at 50 kV or the Philips 200 electron microscope at 60 kV.

RESULTS

Suitable concentrations of all the cationic polymers produced an assortment of morphological changes limited to the gall bladder epithelium. The effects of the several cationic polymers were similar and can be considered under three major categories: (*a*) changes in the apical border of the cell, (*b*) changes in the lateral border, and (*c*) changes in cell volume and integrity. Taken together, these observations suggest a major role for anionic sites in maintaining both the anatomical form and physiological integrity of the cell.

Effect of Polymeric Cations

APICAL BORDER: In controls (Fig. 1), the apical surface is composed of microvilli which

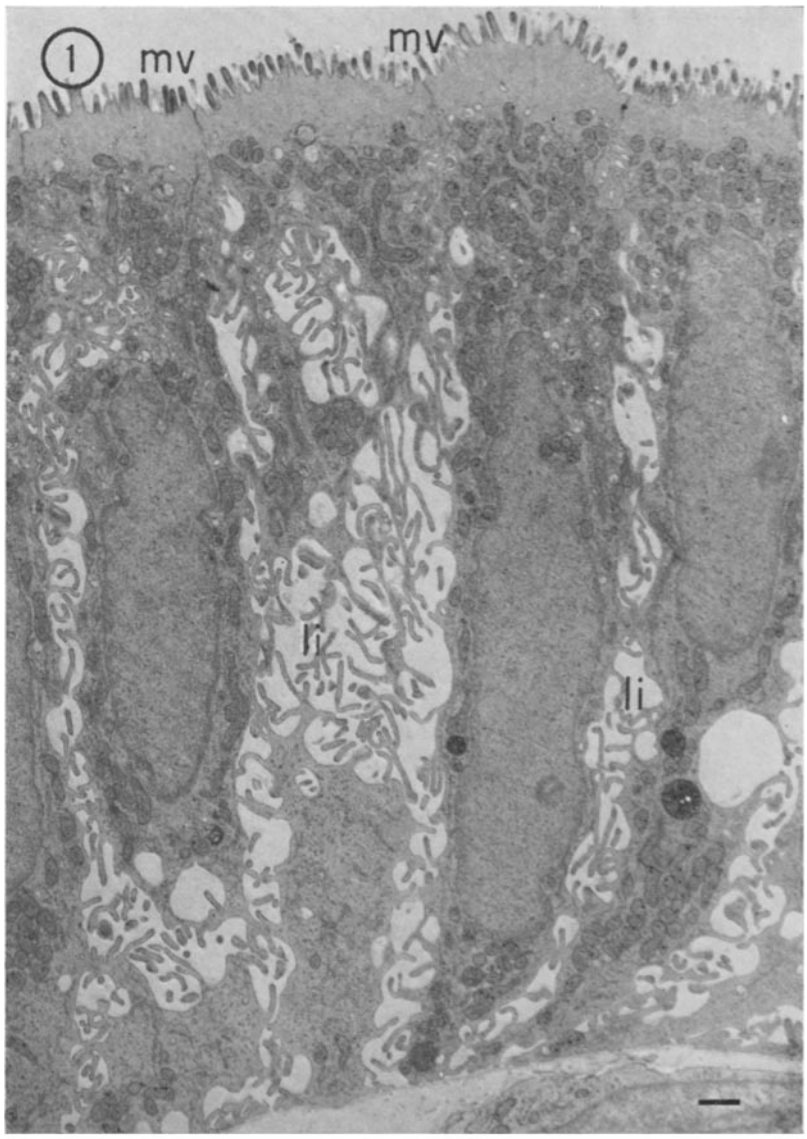

FIGURE 1 Control gall bladder epithelium not exposed to cationic polymers. Note the structural individuality of the microvilli (*mv*) and lateral invaginations (*li*). The cytoplasmic ground substance, mitochondrial size and ground substance, and nuclear size and ground substance are comparatively consistent from one cell to another. Scale bar, 1 μm; 5,300 diameters.

are usually uniform in size and shape, and which are rather evenly distributed along the surface. The uniform upright posture of the microvilli suggests a certain rigidity. Furthermore, the membranous surfaces of even closely adjacent microvilli are never seen to contact one another directly.

Alterations are found at poly-L-lysine concentrations as low as 1.0 μg/ml and at concentrations of 10.0 μg/ml of protamine and histone. (In general, the effects of poly-L-lysine, mol wt 175,000 and of poly-L-lysine, mol wt 2,800, are very similar on a weight-for-weight basis.) The microvilli appear to lose their structural rigidity, and in scattered areas they collapse onto the apical surface. The resulting appearance is one

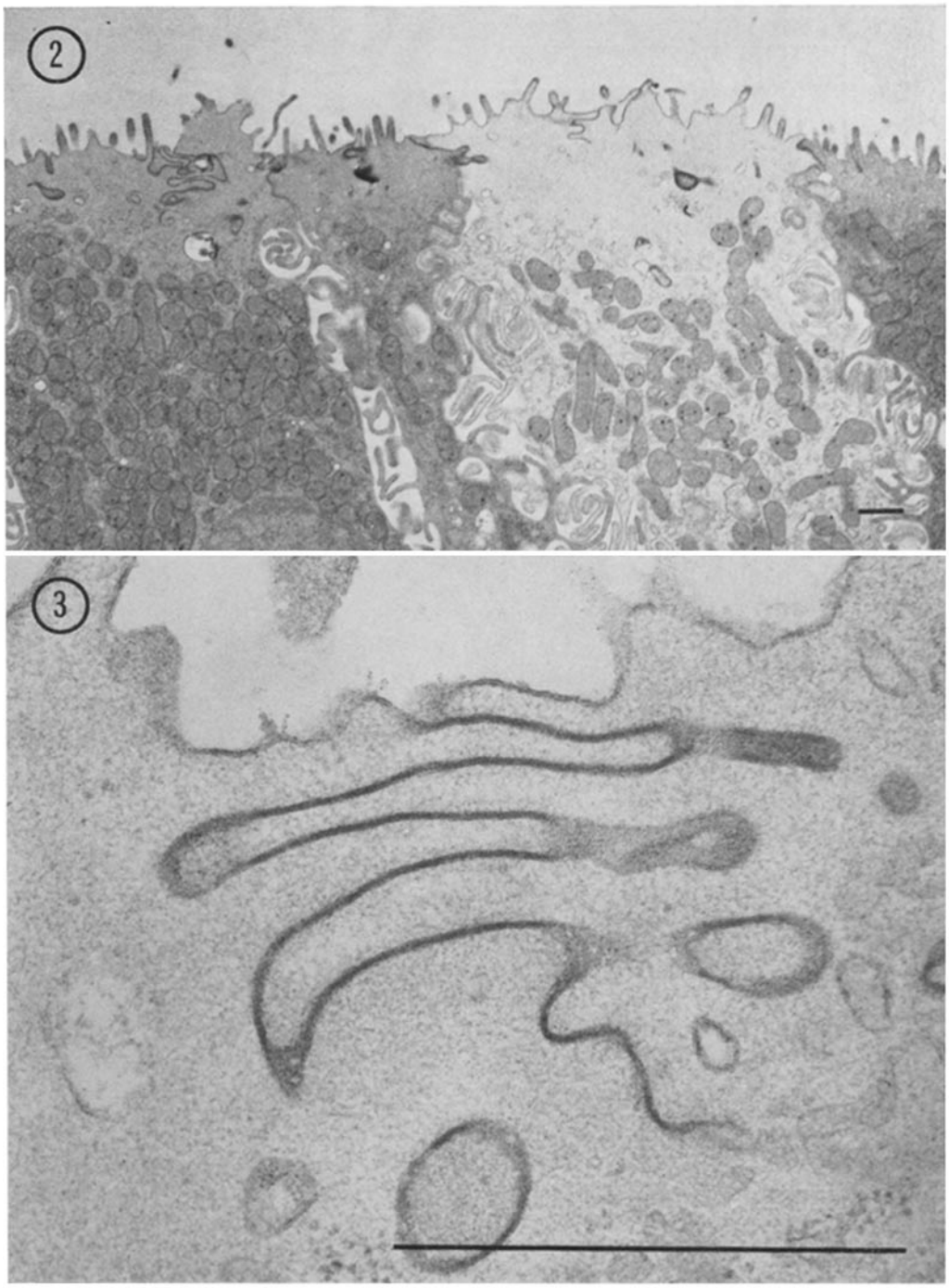

FIGURE 2 Gall bladder epithelium exposed to 1.0 μg/ml of poly-L-lysine. Note: (*a*) the collapse of the microvillar structure, (*b*) the membrane folds extending from the apical border into the terminal web, and (*c*) the contrasting densities of the cytoplasmic ground substances of adjacent cells. Scale bar, 1 μm; 6,600 diameters.

FIGURE 3 Pentalaminar membrane folds formed by plasma membranes exposed to 10.0 μg/ml protamine. Scale bar, 1 μm; 73,000 diameters.

of convoluted sheets of apparently fused membranes extending well into the terminal web region (Fig. 2).

Micrographs of these "fused" membranes (Fig. 3) reveal a pentalaminar substructure reminiscent of tight junction complexes at similar magnification. The significance of the middle dense line is uncertain. It could represent the actual fusion of the outer lamellae of two apposed unit membranes, or it could simply be a very intimate juxtaposition. (Similar images have been reported recently in gall bladder epithelium under certain osmotic conditions, cf. Smulders et al., 1972.)

Poly-L-lysine concentrations of 10 μg/ml cause increasingly extensive and severe aberrations. At the higher concentrations, the microvilli are completely missing from many regions, and are grotesquely distorted elsewhere (Fig. 4). Coiled sheets of membrane are often found along (Fig. 4) the apical border (Fig. 5). Similar membrane alterations are observable in the micrographs of toad bladder epithelium exposed to poly-L-lysine (Mamelak et al., 1969).

LATERAL BORDER: The lateral border of untreated cells is characterized by extensive lateral folds or processes (Fig. 1). These membrane forms differ from the microvilli in being longer, thinner, less uniformly distributed, and apparently more flexible.

The effects of cationic polymers at the lateral borders are qualitatively similar to those at the apical border, although less dramatic. At lower concentrations of cationic polymers, the characteristic membrane "fusion" occurs in the lateral spaces, as adjacent membrane folds are bound together (Fig. 6). Membranes proximal to the tight junction region appear most susceptible to fusion; consequently, the distinguishing hallmarks of the tight junction are frequently obscured in the length of closely juxtaposed membranes. This phenomenon is rare at low concentrations of cationic polymers, but occurs frequently at 100 μg/ml.

Poly-L-lysine (mol wt 2,800) and protamine demonstrated a markedly greater tendency to cause lateral border changes than poly-L-lysine (mol wt 175,000) and histone. This might reflect

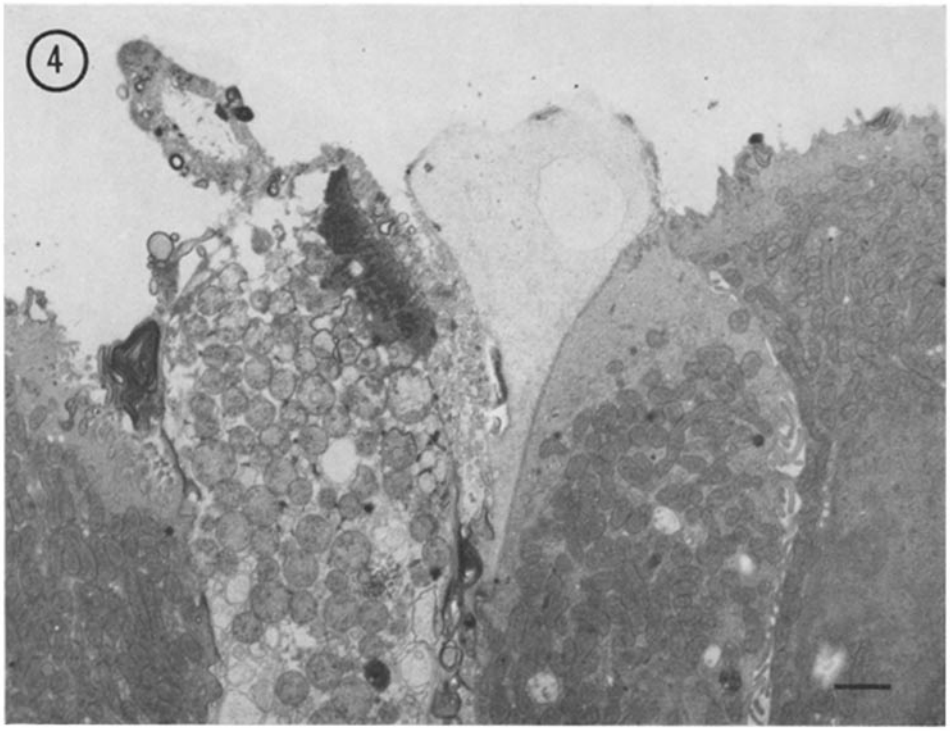

FIGURE 4 Epithelial tissue exposed to 100 μg/ml of poly-L-lysine. The normal morphological patterns of the apical and lateral membranes are destroyed or extensively altered. Scale bar, 1 μm; 7,500 diameters.

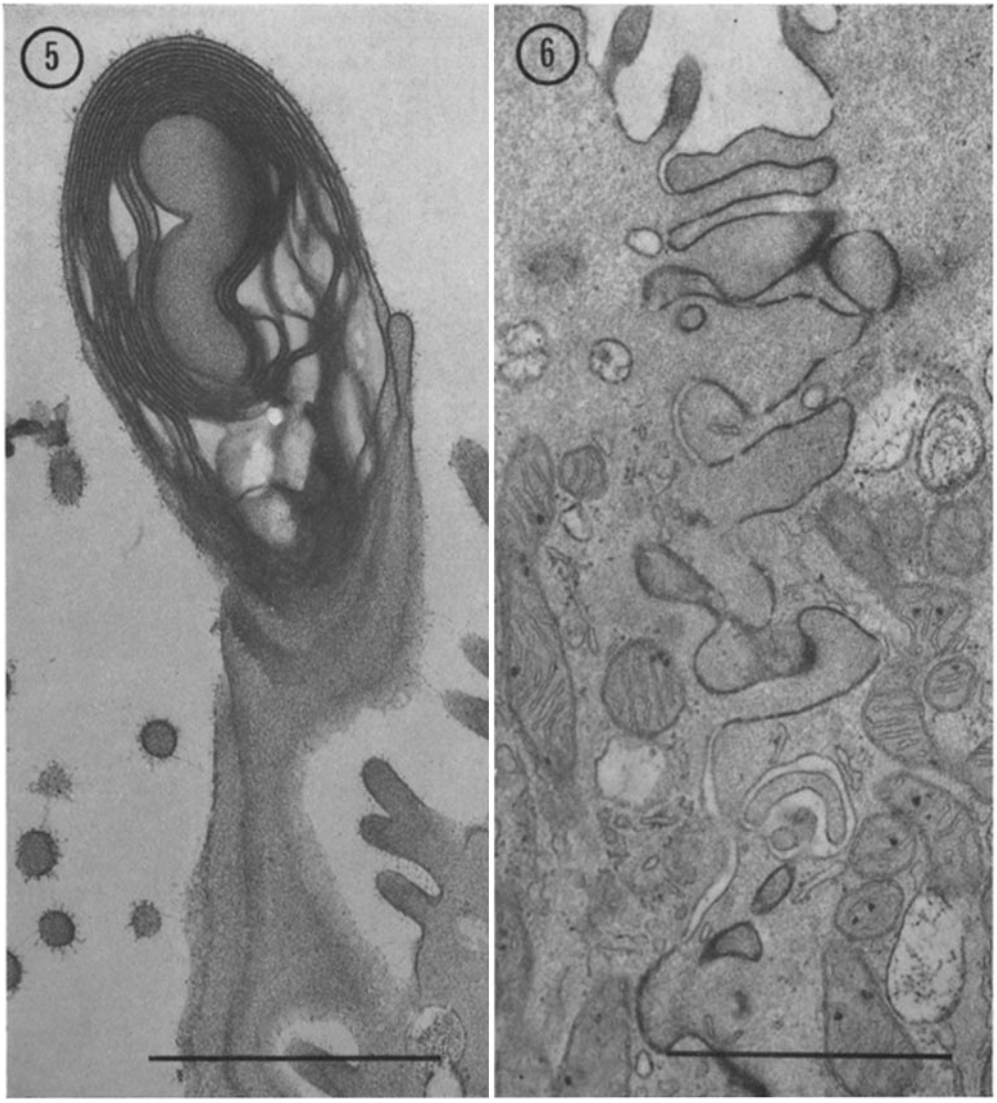

FIGURE 5 A coiled sheet of cell membrane commonly observed after exposing the tissue to 10 μg/ml and 100 μg/ml of cationic polymers. Close examination reveals a pentalaminar structure in each membrane sheet in this case. Scale bar, 1 μm; 39,000 diameters.

FIGURE 6 Fusion of juxtaposed membranes of the lateral spaces induced by 10 μg/ml of protamine. Note that the region of the tight junction is no longer discernible. Scale bar, 1 μm; 38,000 diameters.

the likelihood that the lower molecular weight polymers reach the lateral spaces more readily.

CELL VOLUME AND INTEGRITY: Untreated gall bladder epithelium is composed of cells which are rather uniform in their size and shape. The densities of the cytoplasmic and nuclear ground substance are appreciable and quite uniform from cell to cell. The cytoplasm contains numerous, uniformly compact mitochondria (cf. Fig. 1).

Even at 1.0 μg/ml of poly-L-lysine, cells occasionally show signs of osmotic abnormality (Fig. 7). Their cytoplasmic matrix is less dense than normal (cf. Fig. 2) and their cell margins appear stretched. Likewise, their mitochondria are distended and distorted. Such an appearance is

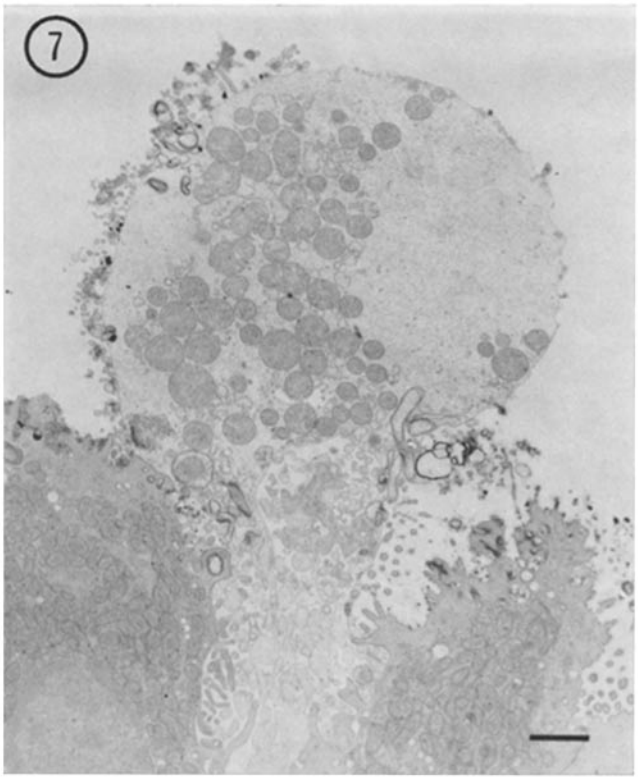

FIGURE 7 An epithelial cell osmotically traumatized due to the effect of poly-L-lysine on membrane permeability. The ground substance appears diluted, the mitochondria are swollen, and the plasmalemma is stressed. Scale bar, 1 cm; 7,800 diameters.

readily interpretable as due to alterations in the properties of the cell membrane and subsequent loss in the ability of the cell to maintain its osmotic integrity.

Higher concentrations of poly-L-lysine and other cationic polymers increase the extent and severity of this phenomenon. Some cells are lysed. At all concentrations, cells at the tips of the mucosal folds are more susceptible to alterations than the cells in the crypts between the folds. Aside from the simple explanation that cells in the crypts are less exposed, the difference in susceptibility may also be related to the pattern of cell replication in the gall bladder (Kaye et al., 1966).

Effects of Heparin

Heparin, taken as an example of a polymeric anion, caused no detectable changes in gall bladder epithelium exposed to it at concentrations of 200 μg/ml.

DISCUSSION

The membrane surface of most, if not all, cells bears a predominant net negative charge (Cook, 1968; Passow, 1969; Sherbet, 1972; Weiss, 1970; Winzler, 1970), which is due to the presence of anionic radicals covalently fixed to the molecular matrix of the membrane (Cook, 1968; Pigman and Gottschalk, 1966; Trinkaus, 1969; Weiss, 1970). Fixed anionic radicals are thought to be present as carboxyls (Rosenberg and Einstein, 1972; Kemp, 1968), sulphonyls (Ito and Revel, 1964), and phosphatyls (Dowben, 1969; Allen et al., 1971). Even though the presence and assumed importance of the fixed anions is undisputed, the exact function of these components in membrane chemistry is not well understood. The present study examines the role of fixed anions by experimentally altering the state of the anion in the membrane *in situ*.

The facts that (*a*) the dominant common chem-

ical property of all the polymeric cations used in our study is a large net positive charge, (*b*) heparin, a polymeric anion with a large net negative charge, had no effect on the membrane, and (*c*) all of the polymeric cations induced very similar alterations in the membrane, strongly suggest that the effects of the polymeric cations are due to a nonspecific interaction (neutralization) with the membrane-fixed anions. The observed effects of neutralizing the fixed anions further suggests that these anions are of fundamental importance in determining membrane organization with respect to at least three distinct membrane phenomena, i.e., membrane-to-membrane interactions, membrane structural rigidity, and membrane permeability.

Membrane-to-Membrane Interactions

The most outstanding effect of polymeric cations at low concentrations is that of inducing fusion of adjacent membranes. Membrane fusion is related most probably to two effects of the interaction: (*a*) neutralization of the negative surface charge on the membrane, and (*b*) formation of polymeric cation bridges which cross-link opposed membrane surfaces (Katchalsky et al., 1959). Complete or partial neutralization of the membrane surface charge is indicated as prerequisite to fusion, since the effectiveness of a polymeric cation to cause fusion is related to its positive charge density, i.e. poly-L-lysine > protamine > histone, while heparin showed no effect at all. It appears, then, that ionized anions on the membrane set up repulsive surface potentials along the membrane surface which establish a minimum distance of approach between surfaces and prevent fusion of juxtaposed membranes. Such phenomena may be particularly crucial in certain membrane configurations such as microvilli, lateral processes, or basal infoldings. The possibility that similar forces are at play throughout the cell in other membrane structures cannot be ignored.

On the other hand, the fact that polymeric cations can join membranes indicates that fixed anions may participate in forming cross-links between fused membranes found in biological systems, e.g., myelin sheaths, junctional complexes, synapses, etc. Although the polymeric cation-induced fusion does not dictate that membranes are fused in nature accordingly, it does present a concrete example of membranes fused by a mechanism argued previously (Humphreys, 1965; Franke et al., 1971; Trinkaus, 1969).

Structural Rigidity

The altered morphology of the membrane processes such as microvilli is open to interpretation. The most ready explanation is that the membrane loses rigidity (stiffness) and becomes more pliable after interacting with a cationic polymer. It seems fair to conclude that the cationic polymers compete for fixed anions and disrupt ionic and hydrogen bonds which normally impart a degree of stiffness to the membrane. The idea that at least part of the membrane should be viewed as a continuous matrix of interlinked molecules has received some support (Green, 1971; Vanderkooi and Green, 1970). However, the mechanism by which matrix molecules are interlinked is ill defined. Fixed anionic sites are well suited for such a cross-linking role by (*a*) binding with membrane-fixed cationic sites (Trinkaus, 1969), (*b*) coordinating with metallic cations (Weiss, 1970; Rappaport and Howze, 1966), and (*c*) forming hydrogen bonds with other electronegative centers. If membrane-fixed anions function in such structural roles, it would be expected that interacting the membrane anions with an extraneous cation could break intermolecular bonds and allow larger degrees of molecular freedom in the membrane, a consequence of which would be a loss of overall membrane rigidity.

The possibility that the changes in morphological forms is due to polymer interactions with underlying structural elements in the cytoplasm seems unlikely, at least in the case of high molecular weight poly-L-lysine, since its size should limit its penetration through the membrane and since a smaller molecular weight poly-L-lysine was localized autoradiographically at the apical membrane almost exclusively (Mamelak et al., 1969).

Permeability

The observation that some cells lose their osmotic balance and swell indicates that the permeability of the cell membrane is substantially increased. An alternate interpretation is that the active transport is inhibited by the interaction; however, tissues incubated in the cold, with ouabain, or with cyanide (conditions which

presumably inhibit transport without altering permeability significantly), induce cell swelling at a much slower rate than observed here (Tormey and Diamond, 1967). If the view is taken that the membrane permeability is ultimately a function of the organizational complex formed between the carbohydrate, protein, and lipid components of the membrane, it seems reasonable to expect that perturbing the organization of any component may have serious repercussions on the membrane organization as a whole and may, therefore, be reflected in permeability changes.

The explanation for the increase in cell membrane permeability induced by cationic polymers is based on the rationale given above with respect to the function of fixed anions and membrane rigidity. Indeed, if intermolecular cross-linking involving fixed anions is critical to molecular rigidity, it is only to be expected that it should be critical to molecular organization and, hence, permeability properties. Further support of these interpretations is derived from recent observations that poly-L-lysine increases the conductance of anionic lipids by two orders of magnitude; the change in conductance is believed to be due to a reorganization of the membrane substructure (Montal, 1972). However, it is not clear how much of the induced reorganization is due to the polymeric nature and other properties of the interacting cation and how much is due simply to the neutralization of the anions in the membrane. It may be that the disruptive effect of the polymers is due at least in part to properties other than cationic charge. It does not seem likely, in view of the relatively large free energy change associated with the electrostatic interaction expected between the polymer cationic elements and membrane anionic elements, that other interactions involving the side chains should contribute significantly to the effect observed here. It is clear, in any case, that if intermolecular interactions (cross-linking) in the membrane involving fixed anions are critical to molecular rigidity and membrane stability, they will also be crucial to molecular organization and, hence, permeability properties.

In conclusion, the results of the present experiments are most readily explained by assuming that membrane-fixed anions maintain important functions in forming both intra- and intermembrane cross-links. This is not to suggest, by any means, that the singular function of the fixed anion is structural, but rather, it is to call attention to a function performed by membrane-fixed anions fundamental to cells in general.

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