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EFFECTS OF POLYMERIC CATIONS ON THE MUCOSAL EPITHELIUM OF RABBIT GALL BLADDER AND A POSSIBLE ROLE FOR THE ANIONIC SITES OF CELL MEMBRANES

By

Paul Marquis Quinton

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

Thesis Director's signature:

Charles W. Plappert

Houston, Texas

May, 1971
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My wife, Bonnie, deserves more gratitude for her patience and understanding than I can express.

As we part, I realize an emotional appreciation, unbecoming to the objective approaches of science, for my laboratory brothers Karl, Pete and Steve.

Thanks is due, also, to Mrs. George Ann Waits for her good cheer and technical assistance as well as to Mr. Bruce Hughes for his efficiency, concern and willing help.

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Introduction

Historical Notes

For biologists, the cell perifery is, and has been, a source of immense intrigue as well as frustration for well over half a century. The presence of some barrier between the cell cytoplasm and its immediate external environment was recognized near the end of the last century. Overton (1895) noted that rates of plasmolysis reversal induced by various compounds usually were related to the solubility of these compounds in oils. During the first quarter of this century, Gorter and Grendel (1925) reported that the lipid extracted from erythrocyte ghosts was sufficient to cover twice the area of the surface of the intact erythrocyte. This observation led them to postulate that the cell was surrounded by a bimolecular lipid layer, i.e. a lipid film two molecules thick. A decade later, Danielli and Davson (1935) postulated the paucimolecular bilayer lipid membrane theory as the basis of cell membrane structure. This hypothesis presented the cell membrane as a symmetrical lipid bilayer with protein distributed on each side between the aqueous phase and the central hydrocarbon element. Meanwhile, by mechanically removing the plasma membrane, Chambers (1940) demonstrated that the
cell could regenerate the membrane and that the membrane was critical in controlling the movement of molecules into the cytoplasm.

With the advent and improvement of the electron microscope for use in biology, Robertson (1955) demonstrated an array of electron dense-light-dense lamellae which he proposed to represent the basic elements of the membrane. From these observations, he presented the "unit membrane" theory which essentially accepted the paucimolecular hypothesis, but went on to state that the membrane possessed an assymetric character and that the central lipid phase was exactly two molecules thick. The paucimolecular bilayer lipid theory of cell membrane structure, even though it has been modified a number of times to account for various phenomena, has possibly received wider acceptance than any other membrane structure hypothesis. On the other hand, seemingly very different ideas concerning the molecular organization of the membrane have been proposed and vigorously defended (Branton 1969, Hendler 1971, Stoeckinius and Engleman 1969). For example, the micellar structure theory (Lucy 1964) proposes that the membrane is composed of protein coated lipid micelles, while the semi-micellar structure theory (Luzzati and Husson 1962) assumes an equilibrium between the bilayer and micellar forms.
Surface Material

Chemical description:

It is as naive as it is difficult to assign dimensions and quantitative compositions to the plasma membrane (Weiss 1963). But since a starting point for a definition of the external surface is convenient, it is suggested (Weiss 1963) that peripheral substances external to the lipoid phase be considered surface material. That such substances exist was postulated early (Chambers 1940) and clearly demonstrated with the electron microscope as filamentous-like material extending from microvilli of mouse gall bladder (Yamada 1955). A few years later, Bennett (1963) proposed that surface associated materials were ubiquitous to cell membranes, and he introduced the term "glycocalyx" to emphasize his belief that carbohydrates constitute a significant portion of the material.

Partially due to the fact that surface materials have not been isolated in convincingly pure preparations, and partially due to their complex structure, their exact chemical composition remains both ill defined and poorly understood. Hirst (1948) first suggested a polysaccharide might be associated with the exterior of the red cell membrane. More specifically, the carbohydrate moiety appears in combination with protein at the cell surface as
a universal characteristic (Martinez-Palomo 1970, Benedetti and Emmelot 1967, Maddy 1969, Stein 1967, Weiss 1963, Winzler 1970). As a result of the lack of definitive chemical information, a rather ill-defined group of terms such as glycoproteins, mucopeptides, mucoproteins, etc., have been introduced to refer to the chemical nature of the surface materials. It is apparent that all of these terms indicate a carbohydrate moiety associated with protein (Ginsburg and Neufeld 1969). In general terms, protein represents about 50% of the total membrane, carbohydrate constitutes less than 10% and lipid represents the remainder (Cook 1968, Korn 1969, O'Neil 1964, Rogers and Parkins 1968, Shimizu and Funakoshi 1970). It should be emphasized that these figures are likely to vary considerably from cell type to cell type (Martinez-Palomo 1970, Shimizu and Funakoshi 1970, Weiss 1970).

Despite the general chemical ambiguity of the surface material, it is well accepted that cell surfaces behave as polyelectrolytes—in particular polyanions (Dowben 1969, Singer and Tasaki 1968). This property has received much attention and has been demonstrated repeatedly. During electrophoresis, erythrocytes, bacteria, and culture cells move to the anode (Heard and Seaman 1961, Katchalski et al 1953, Passow 1969, Weiss 1965). The application of

Fixed anionic sites:

Generally, it is accepted that the polyanionic character of the cell surface is due to fixed negatively charged sites (Cook 1967). The principal source of such sites in biological molecules are limited to three types of anions: phosphates, sulfates, and carboxylates (Mahler and Cordes 1966). Phosphate groups are easily demonstrated as a characteristic component of phospholipids in membrane preparations (Van Deenen 1969, Dowben 1969). However, the net contribution of these groups to the total surface charge is probably small (Dawson 1968, Weiss, 1970), since at physiological pH lecithin is neutral and the cephalins are only slightly negative (Dawson 1968). Of phospholipids, the most negative should be phosphatidyl serine, but it seldom represents more than 10% of the total membrane lipid, (Rouser et al. 1969). Furthermore,
the charged groups of the membrane are probably extensively neutralized by their ionic interactions with the surface proteins (Dawson 1968, Few 1957, Haydon and Taylor 1963, Sessa and Weismann 1967).

Sulfate esters may be involved in the chemistry of surface material since they are detectable in secretory products which may become associated with the surface (Ito and Revel 1964). In general, however, sulfate radicals seem to be absent or contribute little to the surface material (Cook 1967).

It is not only by process of elimination that the carboxyl group seems responsible for the major portion of the surface charge; substantial evidence supports this view. The carboxyl is frequently linked to a hexose base in the form of N-acetyleneuraminic acid (Bendetti and Emmelot 1967, Cook 1968, Cook et al 1961, Heard and Seaman 1961, Martinez-Palomo 1970). Since the acid group can be removed either by trypsin or neuraminidase treatment in a number of systems, it is likely that the acid represents the terminal hexose of an oligo- or poly-saccharide which is covalently linked to a protein in the matrix of the surface material (Marikovsky and Danon 1969, Pigman and Gottschalk 1966, Price 1970). Attempts to quantify the number of negative sites per unit area by
using neuraminidase digestion, calculations of the surface potential from electrophoretic mobilities, and from electrophoretic mobilities of partially neutralized surfaces produce estimates of one site per 250 Å² to 1,800 Å² (Cook et al 1961) (Nevo et al 1955). The difficulty in perfecting such data probably stems from the limitations that 1.) the surface is not a perfect interface, 2.) negative sites may be some distance away from the interface, and 3.) not all forms of the acid are enzyme susceptible (Cook et al 1961, Ravetto 1968). It is nonetheless apparent that surface material is negatively charged.

In summary, the apparent chemistry of the surface material consists of a number of fixed anionic sites that are covalently bound to an oligo- or polysaccharide which in turn is covalently linked to protein. This "unit" forms a matrix associated with the polar heads of the underlying phospholipids. The nature of the protein-lipid association is still a matter of considerable dispute (Branton 1969, Colacicco et al 1967, Eley and Hedge 1956, Haydon and Taylor 1963, Korn 1969).

Question and Rationale of the Study

Question:
Although a number of functions have been suggested for the negatively charged surface, an explicit molecular mechanism by which these charges perform an essential role in cell membrane structure and function has not been presented. Some of the functions that have been proposed to involve the electrostatic or polyanionic nature of the surface are mentioned here: 1.) cell adhesion and aggregation (Kemp 1968) 2.) membrane integrity (Glaeser and Mel 1966, Weiss 1961, 1965, 1970) 3.) cell recognition (Glaeser and Mel 1968) 4.) nerve excitability (Tasaki and Singer 1968) 5.) cation transport (Glick and Glithens 1965, Weiss and Levinson 1969) 6.) antigen - antibody response (Hirst 1948). Furthermore, it is speculated that the polyanionic nature of the surface material may contribute microbuffering capacities to the cell when it is exposed to pH, osmotic, or mechanical shock (Katchalsky 1964, Philpott 1968). It has also been suggested that the surface material may serve as an ion discriminator or molecular sieve (Wright and Diamond 1968, Eisenman et al 1967, Mohos and Skoza 1969). Even though such a number of functions have been postulated for the polyanionic surface, the molecular mechanism by which these functions occur is generally lacking.
Rationale:

If anionic sites of a similar nature are common to cell surfaces, it seems that 1.) there may be underlying molecular conditions which relate the fixed anionic sites to the surface functions, and 2.) a description of that condition may enhance the understanding of cell surface phenomenae. It was therefore the purpose of this study to describe and eludicate, at least to some degree, experimentally induced interactions of the cell surface. The experimental approach carried out in this study consisted of the following: 1.) selecting a membrane system with as many easily measurable parameters as possible, 2.) selecting compounds which would be expected to interact selectively with the surface material, and 3.) assaying the changes in the system parameters due to the interaction of the surface with the selected compounds.

Justification of the Experimental System

Membrane:

The luminal epithelium of the rabbit gall bladder was selected as the source of membrane for the study. Some of the features of the rabbit gall bladder which make it attractive for the present purposes include the following:

Accessibility—In the rabbit, the gall bladder is
large enough (ca. 0.5 x 2.0 cm.) so that excision is only a minor problem and the possibility of accidental trauma is thereby reduced. Furthermore, New Zealand White rabbits are a common breed which are available and represent some genetic consistency.


Assayable parameters—Due to the features described above, the gall bladder preparation presents a number of readily assayable parameters, those which were of concern in the present study are justified below:

1.) Active transport: The transport of fluids across the gall bladder wall in vitro is well documented and is easily measured gravimetrically (Diamond 1964, Dietschy 1964, Frederiksen and Leyssac 1969).

3.) Conductivity: The overall ion conductivity is measurable by passing a small current across the membrane while recording the voltage and current relationships (Diamond and Wedner, 1969, Wright and Diamond 1968).

4.) Morphology: The morphology of the normal rabbit gall bladder has been well characterized (Dietschy 1966, Frederiksen and Leyssac 1969, Kaye and Lane 1965, Kaye et al 1966a, 1966b, Tormey and Diamond 1967).

Compounds:

To study the fixed charge in relation to the surface material, a compound was sought which (at least in theory) would react preferentially with the anionic sites without inducing covalent alterations. Methods inducing covalent changes such as enzymatic digestion (Weiss 1961, Cook et al 1961) or reagent reactions (Heard and Seaman 1961, Berg et al 1965) are certain to alter molecular architecture that may have little to do with the anionic sites. Therefore, a compound which would interact solely on an electrostatic basis was sought. A compound with a large molecular weight was preferred as a precautionary measure against membrane penetration which might cause complicating cytoplasmic interactions. It should also have a well defined molecular structure to minimize any ambiguities.
as to the nature of its interaction. Furthermore, a compound with a "biological history" was preferred in order to compare and substantiate its interaction in the present system with those previously reported in other systems.

1.) Poly-L-Lysine, molecular weight 175,000 (P.L. 175,000)-- P.L. 175,000 met the above experimental requirements well. It is readily synthesized in the laboratory from -N-Carboxy-L-lysine anhydride (Katchalski and Sela 1958). Its molecular structure is well defined, having a positively charged primary -amine terminating the side chain of each lysine monomer (Katchalski et al 1964). Furthermore, it may be obtained as a high molecular weight polymer consisting entirely of lysine monomers (Applequist and Doty 1962). Thus, at physiological pH, the polymer is a random coiled, highly charged cationic polyelectrolyte having a high molecular weight and a well defined structure (Applequist and Doty 1962).

Furthermore, the polymer has a substantial "biological history". It is known to inhibit bacterial growth and virus infectivity (Buchanan-Davidson et al 1960, Katchalski et al 1952, Rubini et al 1951). It acts antagonistically to heparin, and is a strong agglutinating agent with red
cells and bacteria (DeVries et al 1951, Katchalski et al 1953, Katchalsky et al 1959, Nevo et al 1955). The polymers act to precipitate a number of biological compounds including plasma albumin (Rice et al 1954), heparin (Rubini et al 1951), nucleic acids (Matsuo et al 1969, Matsuo and Tsuboi 1969) and the soluble components of the red blood cell (Danon et al 1965). More to the point of this study, P.L. polymers produce alterations in such membrane systems as chloroplasts (Dilley 1968), mitochondria (Swartz 1965) and, pinocytosis in tumor cells and amoebae (Kornguth et al 1961, Ryser 1967, Ryser et al 1965, Sanders and Bell 1970). It increases the efflux of potassium and betacyanin from beet cells (Osmond and Laties 1970, Siegal and Daly 1966), and affects ion transport and/or membrane permeability in toad bladder (Mamelak et al 1969).

Three other cationic polymers were selected to compare their action to that of P.L. 175,000 for the reasons below.

2.) Poly-L-Lysine, molecular weight 2,800 (P.L. 2,800)--A low molecular weight homopolymer analogue of P. L. 175,000 was selected to determine if there were any idio-cyncratic behaviors peculiar to larger molecular weight polymer.
3.) Protamine--The compound is a highly charged cationic polymer having a molecular weight in the neighborhood of 5,000. Arginine is believed to account for at least 70 to 80% of its total amino acid composition, hence its high positive charge (Mahler and Cordes 1966). In a number of systems, protamine has properties similar to those of P.L. For example, it is an anti-heparin agent (DeVries et al 1951, Rubini 1953), and agglutinates red blood cells (Katchalsky et al 1959), stimulates the release of betacyanin from beet cells (Siegal and Daly 1966), and stimulates phosphorylation in liver mitochondria (Swartz 1965).

4.) Arginine rich histone (histone)--Histone, also, is a biologically synthesized cationic compound; although, it is generally more heterogeneous than protamine and usually has a molecular weight range from 10,000 to 20,000 (Mahler and Cordes 1966). Aside from its arginine content, histone has a significant lysine, as well as glutamate and aspartate, content (Murray 1964). The latter two are anionic residues which clearly make histone a heterogeneous acid-base polyelectrolyte. Its net positive charge stems from the fact that there are more basic than acidic residues (Murray 1964). Nonetheless, the biological effects of this
compound are reported somewhat similar to those of P.L. It stimulates oxidative phosphorylation in mitochondria (Swartz 1965, Johnson et al 1967). It stimulates the apparent pynocytotic activity in sarcoma cells (Ryser and Hancock 1965). And like P.L. and protamine, it blocks sodium reabsorption in the rat parotid (Mangos and McSherry 1968).

The biologically synthesized proteins described above were employed in some experiments to determine if they exhibit interaction properties similar to those of P.L. and, if so, to facilitate speculation of the in vivo role of such compounds.

Objectives of the Study

This study proposes to accomplish the following objectives:
1.) to assay the effect of polymeric cations (P.C.), emphasizing the effect of P.L. 175,000 on fluid transport in vitro as a preliminary criterion for P.C. - surface interaction.

2.) to examine the effects of minimal concentrations of P.L. 175,000 on the membrane ion perm-selectivity patterns.

3.) to examine the effect of minimal concentrations of P.L. 175,000 on the overall ion conductivity of the membrane.
4.) to examine the morphological effects of several concentrations of P.C. on the gall bladder mucosal epithelium.

5.) to attempt a formulation of a molecular mechanism by which induced alterations may be explained and, if possible, to extend the interpretations to the functional roles of fixed anionic sites in cell surfaces.
## Table I. Composition of Physiological Solutions

The names of solutions as they appear in the text are listed in the first column. The corresponding concentrations in millimoles of each of the solution constituents are given in the succeeding columns.

<table>
<thead>
<tr>
<th></th>
<th>NaCl</th>
<th>KCl</th>
<th>LiCl</th>
<th>CaCl₂</th>
<th>MgSO₄</th>
<th>Glucose</th>
<th>NaH₂PO₄</th>
<th>Na₂HPO₄</th>
<th>NaHCO₃</th>
<th>Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-Ringer's</td>
<td>150</td>
<td>7</td>
<td>-</td>
<td>1.0</td>
<td>1.2</td>
<td>11.1</td>
<td>.375</td>
<td>2.125</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2 Na-Ringer's</td>
<td>75</td>
<td>7</td>
<td>-</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-Ringer's</td>
<td>-</td>
<td>157</td>
<td>-</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li-Ringer's</td>
<td>-</td>
<td>7</td>
<td>150</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td></td>
<td></td>
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<tr>
<td>Bicarbonate Ringer's</td>
<td>110</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>1.2</td>
<td>&quot;</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>10x Ca-Ringer's</td>
<td>150</td>
<td>&quot;</td>
<td>10.0</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>.375</td>
<td>2.125</td>
<td></td>
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</tr>
</tbody>
</table>
Materials and Methods

Materials--General

Animals: Gall bladders were obtained from New Zealand White Rabbits weighing between 2.5 and 4.0 kilograms. Rabbits were maintained on Purina Rabbit Chow supplemented with lettuce.

Polymeric cations: Poly-L-lysine HBr (lot # 78B-0890), poly-L-lysine HBr (lot # 59B-5010), heparin (lot # 18B-0800), and arginine rich histone (lot # 118B-1480) were all secured from Sigma Chemical Company*. Protamine Sulfate (lot # 17952) was secured from K&K Laboratories*.

Physiological solutions: All solutions used contained the same concentrations of essential ions and glucose. Solutions were made to molal concentrations taken primarily from Diamond (Diamond 1964, Machen and Diamond 1970). In the text, solutions are referred by the name of their principal ion constituent as listed in Table I.

P.C. solutions were made by dissolving the compound to a concentration of 100 ug/ml in the appropriate buffer.

*Sigma Chemical Company, 3500 DeKalb St., St. Louis, Missouri.
Lower concentrations were obtained by dilution of the above solution.

Equipment: Graviometric measurements were made with a Roller-Smith Precision Balance with a 500 mg full scale. Transmembrane potentials and D.C. currents were measured with a Bausch and Lomb VOM 7 recording electrometer. The potentiometer circuit of the instrument had a maximum sensitivity of 0.5 mV full scale with input impedance infinite at null. The microampmeter circuit had a maximum sensitivity of 1.0 microamp with impedance of 1000 ohms.

Sample Purity and Homogeneity: Polymeric Cations

In order to confirm the purity and homogeneity of polymeric cations, several assays were performed.

Solubility: Solutions of each P.C. were prepared to total concentrations of 450 ug/ml, 225 ug/ml, and 112.5 ug/ml. The adsorbance of all solutions at 218 mu were recorded on the Zeiss PMQ II Spectrophotometer using matched quartz cuvettes.

Terminal Amine Assay: A modification of the Sanger reaction (Dubin 1960) which was reported as an assay for P.L. (Mamelak et al 1969) was performed on all P.C. Concentrations
of 3.0 uEq/ml were made up in a sodium Ringer's solution. A standard was prepared by dissolving 1.7 uEq/ml of lysine amino acid in the same buffer. Reactions with fluorodinitrobenzene were carried out according to Dubin (1960), and assayed by absorbance at 370 μm when possible. An absorbance assay was not possible when this reaction produced an insoluble precipitate.

Sedimentation: A synthetic boundary cell was used in the Spinco Model E Analytical Centrifuge to determine the homogeneity and approximate molecular weight of P.L. 175,000 (Schachman 1961). P.L. 175,000 was dissolved to a concentration of 0.4% in 1 N NaCl solution buffered to pH 4.5 with 0.02 M sodium acetate (Applequist and Doty 1962). Boundaries were formed at 60,000 r.p.m. at 18.2° C. Boundary displacements were photographed as Schlieren patterns and measured with a Nikon Microcomparator.

Molecular Sieving: A fractionation column of Sephadex G-15, having a lower exclusion limit of 1,500 molecular weight units, was equilibrated with 1 N NaCl buffered to pH 7.3 with 3.0 mM sodium phosphate. A 0.3 ml volume of 20 mg/ml sample dissolved in a 30% sucrose solution was applied to the column and eluted at a rate of 0.2 ml/min.
Fractions were collected in 0.5 ml volumes. The column void volume of 10.5 ml was determined with blue dextran, molecular weight, 2,000,000.

Disc Electrophoresis: Acrylamide gels were prepared after Davis (1964). Stacking gels were made to pH 6.7 with 1 N potassium acetate while separating gels and the anode and cathode buffer solutions were made to pH 4.3 with acetic acid (7.5 mM) plus beta-alanine (30.0 mM). A 20 lambda volume of each P.C. at a concentration of 20 mg/ml was applied to individual gels. Methyl green was used as a tracking dye under a current of 5 mAmps per column. After running, gels were fixed for 10 minutes in saturated picric acid, rinsed in 0.05 sodium barbital, and stained with 0.2% fast green. Destaining was effected by rinsing overnight in the barbital buffer.

Methods—Physiology

Bladder Preparations: The entire cystic lobe of the liver with the bladder attached was removed from rabbits which had been anesthetized with 2 to 4 ml. of sodium pentabarbitol (50 mg/ml) I. V. Bladders were separated from the underlying hepatic tissue by carefully snipping through the connecting tissue. The bladders were then
drained of bile, rinsed everted, and rinsed again in Na Ringer's solution. Bladder sacs which were intended for fluid transport studies or diffusion potential studies were cannulated with a polyethylene tube 0.28 cm O. D. Bladders to be used in conductance studies were cut longitudinally and mounted in a modified Ussing chamber (Wright and Diamond 1968).

Fluid Transport Measurements: Cannulated everted bladders were immersed in bicarbonate Ringer's bathing solutions maintained at 37° C and bubbled with 95% O₂-5% CO₂. At five minute intervals, bladder preparations were lifted briefly from the bathing solution, gently drained on the edge of the bathing solution beaker, and weighed to the nearest milligram. The total weight was plotted on the ordinate against time on the abscissa. The slope of this plot was taken as the average rate of fluid movement. To assay the effect of the P.C., the slope of a fifteen minute period after addition of the given concentration was compared to that of similar, previous control periods. The threshold concentration of P.L. 175,000 effect was determined by dividing the rate during a 30 minute period before the addition of P.L. into the rate during a 30 minute exposure to a given P.L. Since each experiment gave a single quotient (rate during ÷ rate before, P.L.), the
quotients of experiments assaying the same concentrations of P.L. were treated as sample populations. Each sample population was then compared to a control population which had not been treated with P.L. The statistical significance of the comparison was determined by analysis of the difference of two sample means.

Diffusion Potentials: The gall bladder membrane was connected in series by agar-salt solution (KCl Ringer's in Fig. 1). In order to minimize junction potential artifacts, the arrangement depicted in Figure 1 was constructed. The input terminals of the electrometer were connected to calomel electrodes (Sargeant) immersed in saturated KCl solution. As illustrated in Figure 1, the saturated KCl solution in the circuit on the serosal side of the bladder was connected to KCl trap B of Na-Ringer's with a saturated KCl agar-salt (2% agar in all bridges) bridge #4. The KCl trap was then connected to the luminal (serosal) solution through Na-Ringer's agar-salt bridge #3. The serosal solution was in all cases Na-Ringer's. The salt solution (KCl) composing the mucosal bath, agar-salt bridge #2, and KCl trap A depended upon the type of diffusion potential to be measured. For example, if a K:Na bi-ionic diffusion potential were to be evaluated, the mucosal bathing solution, agar-salt bridge #2, and KCl trap A would
all be composed of K-Ringer's. The KCl trap A is, in turn, connected to the mucosal calomel electrode with saturated KCl agar-salt bridge #1. The KCl traps serve to prevent contamination of the serosal and mucosal bathing solution with KCl from the saturated KCl agar-salt bridges which are used to minimize junction potentials.

All bathing solutions were maintained at 37° C and were stirred continuously with magnetic stirrers. The signs of all potentials are recorded with respect to the mucosa.

The effects of P.L. 175,000 at concentrations of 1-5 ug/ml were assayed according to the following protocol. 1.) diffusion potentials for the bladder were recorded after allowing about 1/2 hour for equilibration. 2.) the diffusion potentials were recorded again after the preparation had been exposed to P.L. dissolved in a 10x Ca Ringer's for 1/2 hour. 3.) diffusion potentials were recorded for the last time after exposure to the same P.L. concentration in Na-Ringer's for 1/2 hour. In some experiments the exposure to 10x calcium was omitted.

Membrane Conductance: Bladders were slit open and pinned across a 1.0 cm² opening in a modified Ussing chamber. The chamber was assembled and a circuit arrangement equivalent to that in Figure 1 was adopted. In this case,
however, the mucosal and serosal agar-salt bridges were inserted into the mucosal and serosal halves of the chamber at fixed distances (ca. 0.2 cm.) as close as possible to the bladder surfaces. Current passing Na-Ringer's agar-salt bridges were inserted into the ends of each half of the chamber as distal to the bladder surfaces as possible (ca. 1.5 cm.). The agar-salt bridges each led to a saturated solution of KCl each of which contained a Ag-AgCl electrode. Each terminal of a 9.8 volt Mercury cell was connected to an Ag-AgCl electrode. A variable resistance was inserted between one battery terminal and an electrode. With this circuit, 70 to 100 uAmps were passed through the membrane for brief periods while the trans-bladder potential was recorded. The conductance was calculated from the relation \( C = \frac{I}{V/A} \), where \( C \) = specific conductance, \( I \) = the current passed through the membrane, \( A \) = the cross-sectional area of the chamber opening, and \( V \) = the potential drop across the membrane less the drop without the membrane in place.

All experiments were carried out at 37° C, and continuous stirring was effected with miniature magnetic stirring bars in each chamber.

The effects of 1.0 to 10.0 pg/ml P.L. 175,000 were assayed in an analogous manner to that employed in the
<table>
<thead>
<tr>
<th>ug/ml</th>
<th>P.L. 175,000 5 min.</th>
<th>P.L. 175,000 30 min.</th>
<th>P.L. 2,800 30 min.</th>
<th>Lys. Mono. 30 min.</th>
<th>Histone 30 min.</th>
<th>Protamine 30 min.</th>
<th>Heparin 30 min.</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<td>10</td>
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<td>X</td>
<td>X</td>
<td>X</td>
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<td>X</td>
</tr>
<tr>
<td>1.0</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>0.1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Table II. Morphological Studies: Time and Concentrations
The concentrations of the compounds examined for morphological effect are indicated by an "X" in the appropriate row.
diffusion potential studies. The following protocol was used: 1.) the Conductance was measured after equilibration with Na-Ringer's. 2.) The conductance was measured after mucosal exposure to the selected P.L. concentration in the presence of 10x Ca-Ringer's for thirty minutes. 3.) The conductance was measured after mucosal exposure to the same concentration of P.L. in Na-Ringer's for thirty minutes. All conductances were measured while Na-Ringer's alone constituted the bathing solutions.

**Methods—Morphology**

Tissue Preparation: While gall bladders were excised and rinsed as described above. Bladders were slit open and sliced into small pieces (ca. 2 x 4 mm.) which were pinned by their edges to parafin strips, mucosa exposed. The parafin strips were then transferred to Na-Ringer's solutions containing the appropriate concentration of the selected compound. The concentrations of the various compounds to which tissue was exposed are given in Table II. Except for one experiment, tissue was exposed for 30 minutes to the given concentration. In that experiment, tissue was exposed to concentrations of P.L. 175,000 for only 5 minutes (Table II). All experiments were carried out at 37° C, and the solutions were continuously agitated.
Fixation: After incubation, tissue was rinsed briefly in cold Sorenson's phosphate buffer at pH 7.3. Subsequently, the tissue was fixed immediately with 1% osmium tetraoxide in Sorenson's buffer for 60 minutes at 4°C. During the fixation period, the strips of tissue were diced into smaller pieces (ca. 1 x 1 mm.). Tissue was then dehydrated with ethanol and processed for embedding in Epon 812 according to Luft (1961).

Sectioning: Specimens were sectioned on the Porter-Blum MT-2 microtome. Sections for light microscopy were cut with glass knives to approximately 1 micron thickness. This sections for transmission electron microscopy were cut with a Dupont diamond knife to thicknesses of 600-800 Å.

Staining: Sections for light microscope studies were stained with a combination stain made by mixing in a ratio of 1:1:1, 0.5% Safarin 0, 0.5% Methylene Blue, and 0.5% Azure II, all dissolved in 1% sodium borate. This mixture was derived imperically to give good results in staining the mucosal epithelium of the gall bladder. Thin sections were stained in a conventional manner (Reynolds 1963).

Examination: At the level of the light microscope, sections were examined with a Zeiss Ultraphot under bright field illumination. This sections were examined at the ultra-structure level with either the RCA EMU-3F
at 50 KV or with the Phillips 200 electron microscope at 60 KV.
<table>
<thead>
<tr>
<th></th>
<th>450 ug/ml</th>
<th>225 ug/ml</th>
<th>112.5 ug/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.L. 175,000</td>
<td>1.72</td>
<td>0.91</td>
<td>0.45</td>
</tr>
<tr>
<td>P.L. 3,800</td>
<td>1.60</td>
<td>0.81</td>
<td>0.39</td>
</tr>
<tr>
<td>Protamine</td>
<td>1.80</td>
<td>0.88</td>
<td>0.34</td>
</tr>
<tr>
<td>Histone</td>
<td>2</td>
<td>2</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Table III. Ultraviolet absorbance of Polymeric Cations. The Optical Density at 218 mu of three different concentrations of each polymeric cation is listed.
Results

**Purity of P.C. Compounds**

Solubility: Table III lists the absorbancies at 218 mu of each P.C. at the indicated concentration. The equal absorbance of the two P.L. samples and protamine at equal concentrations, and the variation of absorbance with concentration indicates that these compounds are effectively dissolved at these concentrations. The discrepancy of histone will be reserved for later discussion.

Terminal amine assay: The reaction products of the P.L. polymers were insoluble. The absorbancies of protamine and histone did not agree well with lysine amino acid using the amino acid as a standard. As concluded and discussed in the next section, this assay provided only a qualitative assay of terminal amines.

Sedimentation: A sedimentation coefficient of 0.98 S was determined for P.L. 175,000 (Schachman 1959). Figure 2 shows the boundary formed at 0, 32, and 76 minutes after reaching speed. Inspection of the boundary movement and spread indicated good homogeneity.

Molecular sieving: Over 90% of the P.L. 2,800 sample was eluted with the first void volume. A short, broad peak containing less than 10% of the sample was observed
<table>
<thead>
<tr>
<th>Compound</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.L. 175,000</td>
<td>8</td>
</tr>
<tr>
<td>P.L. 2,800</td>
<td>100</td>
</tr>
<tr>
<td>Protamine</td>
<td>95</td>
</tr>
<tr>
<td>Histone: disc 1</td>
<td>15</td>
</tr>
<tr>
<td>disc 2</td>
<td>49</td>
</tr>
<tr>
<td>disc 3</td>
<td>75</td>
</tr>
<tr>
<td>disc 4</td>
<td>57</td>
</tr>
</tbody>
</table>

Table IV. Disc Electrophoresis Rf Indices for Polymeric Cations. The underlined values represent the major discs of histone. Rf value are given in terms of the tracking dye, Methyl Green.
near the end of the second void volume. Since molecules of over 1,500 molecular weight are excluded from the seive, only a small amount of low molecular weight contaminate is indicated.

Disc electrophoresis: All compounds assayed moved cathodally. P.L. 2,800 and protamine ran at a rate nearly equal to that of the tracking dye. Histone was found in several bands distributed unevenly throughout the disc. The results are recorded in terms of individual Rf's for each P.C. assayed in Table IV. The cathodal movement indicates that all P.C. are positively charged, and the single discs presented by P.L. 2,800 and protamine represent sample homogeneity.

In general, it is felt that P.L. 175,000, P.L. 2,800 and protamine represent samples of polymeric cations which are satisfactorily homogeneous with respect to molecular weight and chemical composition. Histone is accepted as a sample of several distinct cationic molecular species as has been reported previously (Murray 1964).

**Physiology**

Active transport: Increasing the concentration of the P.C. in the bathing solution by an order of magnitude
<table>
<thead>
<tr>
<th>P.L. Conc.</th>
<th>0.0</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
<th>100.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology Changes in 5 min. Exp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Morphology Changes in 10 min. Exp.</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Mean a.r.r.</td>
<td>81.6±15.0</td>
<td>79.4±7.8</td>
<td>57.3±16.1</td>
<td>9.2±9.3</td>
<td>-11.8±8.6</td>
</tr>
<tr>
<td>No. Expts.</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>&quot;p&quot; values</td>
<td>1.0</td>
<td>.75</td>
<td>.025</td>
<td>.01</td>
<td>.01</td>
</tr>
</tbody>
</table>

Table V. Effects of Different Concentrations of P.L. 175,000 on Morphology and Fluid Transport. The extent of morphological alterations is noted by "-" (no change) and by the number of "+"s which subjectively corresponds to the effect of the indicated concentration. The last three rows give the corresponding effect on fluid transport, the first row of which give the mean average remaining rate (see text); the second gives the number of experiments from which the transport data was compiled, and the third gives the probability that each of the P.L. groups belongs to the control population (0.0 ug/ml).
at successive 15 minute intervals causes transport inhibition as reflected by the plots of Figure 3. As reflected by these experimental techniques none of the compounds at 1.0 ug/ml has a detectable inhibitory effect. At 10.0 ug/ml the effects of P.L. 2,800 and P.L. 175,000 are apparent, and even more so at 100.0 ug/ml concentrations.

More rigorous investigations of the effects of P.L. 175,000 were performed in order to estimate the threshold concentration necessary to produce detectable changes in the transport rates (Fig. 4). For this purpose, each group of experiments assaying the same concentration of P.L. was treated statistically as a separate population. Each member of each population was assigned a value which represented the ratio of the transport rate during a 30 minute exposure to a P.L. concentration to the transport rate during the previous 30 minutes. A control population was established by omitting the P.L. during the second transport period. The statistical difference between the mean of the control population and the mean of each of the populations testing a concentration of P.L. were calculated. Since the ratio of the second period to the first period gives the remaining transport rate as a percentage of the original, it is convenient to call this ratio the "average remaining rate" (a.r.r.). Table V correlates
### Diffusion Potentials

<table>
<thead>
<tr>
<th>Expt. #</th>
<th>P.L. conc. mg/ml</th>
<th>2:1 Na</th>
<th>K:Na</th>
<th>Li:Na</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>%</td>
<td>Before</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>13.8</td>
<td>13.1</td>
<td>95</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>12.4</td>
<td>11.5</td>
<td>93.0</td>
</tr>
<tr>
<td>3</td>
<td>0.0</td>
<td>10.0</td>
<td>9.4</td>
<td>94.0</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>12.6</td>
<td>8.4</td>
<td>66.5</td>
</tr>
<tr>
<td>5</td>
<td>1.0</td>
<td>11.4</td>
<td>7.2</td>
<td>63.0</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>13.8</td>
<td>8.6</td>
<td>62.5</td>
</tr>
<tr>
<td>7</td>
<td>2.0</td>
<td>13.3</td>
<td>7.3</td>
<td>55.0</td>
</tr>
<tr>
<td>8</td>
<td>2.0</td>
<td>12.7</td>
<td>7.1</td>
<td>56.0</td>
</tr>
</tbody>
</table>

Table VI. Effect of P.L. 175,000 on Diffusion Potentials. Values for the diffusion potentials in mV of 2:1 Na, K:Na, and Li:Na obtained before and after 30 minutes exposure to the given concentration of P.L. The values obtained after the exposure to P.L. are given as percentages of the value obtained before exposure to P.L. in the column labeled "%".
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Before 10x Ca-Ringer's</th>
<th>After 10x Ca-Ringer's plus 5.0 ug/ml P.L.</th>
<th>After P.L. in Na-Ringer's</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.0</td>
<td>-24.5</td>
<td>1.1</td>
</tr>
<tr>
<td>2</td>
<td>10.5</td>
<td>-31.0</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>10.4</td>
<td>-30.4</td>
<td>0.6</td>
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</tbody>
</table>

Table VII. Protective Effects of Calcium Values of diffusion potentials (mV) are given for bladders before exposure to 10x Calcium concentration, after a 30 minute exposure to 5.0 ug/ml P.L. 175,000 dissolved in 10x Ca-Ringer's, and after a 30 minute exposure to the given concentration (ug/ml) of P.L. dissolved in Na-Ringer's. Parenthesis indicate the listed value as a percentage of the previously recorded diffusion potential of the same type.
the a.r.r. of each concentration of P.L. assayed with detectable morphological effects. There was no significant effect at 0.1 ug/ml. At 1.0 ug/ml the effect was statistically significant; i.e., the mean a.r.r. of the population testing 1.0 ug/ml was significantly less than the mean a.r.r. of the control population at the 0.05 level (p<0.025). The mean a.r.r. of both populations, 10.0 ug/ml and 100.0 ug/ml, were significantly less than the control population, each having a "p value" considerably less than 0.01.

Diffusion potentials: Inspection of the data listed in Table VI shows that the potentials of controls seldom varied more than 10% from the original diffusion potentials. However, after an exposure to 1.0 ug/ml P.L., 2:1 Na diffusion potentials dropped to 60-70% of previous values. K:Na diffusion potentials dropped to 50-60% of their original values and Li:Na potentials increased by 200-260% of previous values. In each case the trend is toward the free solution junction potential. It is for this reason that the change in Li:Na potentials are shown as increases in potential. Increasing the P.L. concentration to 2.0 ug/ml induced slightly greater changes in diffusion potentials.

From Table VII it may be seen that exposing the
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Na-Ringer's</th>
<th>After 10x Ca-Ringer's</th>
<th>After 10x Ca-Ring. plus P.L.</th>
<th>After P.L. in Na-Ringer's</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cond.</td>
<td>min.</td>
<td>Cond.</td>
<td>P.L.</td>
</tr>
<tr>
<td>1</td>
<td>30.99</td>
<td>30</td>
<td>30.18</td>
<td>30.87</td>
</tr>
<tr>
<td>2</td>
<td>32.76</td>
<td>40</td>
<td>32.76</td>
<td>32.32</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
<td>42.12</td>
<td>35</td>
<td>43.15</td>
<td>56.33</td>
</tr>
<tr>
<td>4</td>
<td>26.14</td>
<td>30</td>
<td>27.22</td>
<td>27.79</td>
</tr>
<tr>
<td>5</td>
<td>26.82</td>
<td>30</td>
<td>29.07</td>
<td>28.73</td>
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<tr>
<td>6</td>
<td>23.47</td>
<td>35</td>
<td>23.57</td>
<td>23.57</td>
</tr>
</tbody>
</table>

Table VIII. Effects of Different Concentrations of P.L. 175,000 with and without increased Calcium. The conductances in Na-Ringer's at the times indicated are given for the conditions heading the columns. Controls are given in the last three rows. Conductivities are listed in ohms$^{-1}$ cm.$^{-1}$ x 10$^{-2}$. P.L. concentrations are given in ug/ml.
bladder to 1.0-2.0 ug/ml P.L. in the presence of 10x Ca-
Ringer's produced no significant change in the membrane
diffusion potentials. However, when the 10x Calcium
was removed, changes were apparent. It should be added
that during the course of these experiments (3-4 hours),
the values of these passive parameters held fairly
constant as observed before (Diamond and Harrison 1966).

Conductivity: Addition of 1.0 ug/ml P.L. 175,000 to
the mucosal solution increased the conductivity by approx-
imately 27% average of 3 experiments. In one experiment,
raising the concentration of P.L. to 5.0 ug/ml increased
the conductivity by 52% of its original value. Data for
conductivity changes are presented in Table VIII. It will
be noted that 10x Ca-Ringer's appears to block the P.L.
induced conductance change. This effect is apparently
overcome by increasing the P.L. concentration to 10.0 ug/ml.
The time course of conductance changes in a control and
in an experimental are depicted in Figure 5. It is seen
that 10x Ca-Ringer's did not reverse the conductance
increase induced by 5.0 ug/ml P.L. 175,000.

**Morphology**

Since this study is principally concerned with the
structure of the apical membrane, and since a detailed description of the gall bladder tissue may be obtained from the literature (Kaye et al 1966a, 1966b, Tormey and Diamond 1967, Yamada 1955), only the mucosal epithelium will be described in detail here. The normal morphology of the mucosa is the same as that observed in controls of this study. Therefore, all experimentally induced differences in morphology will be described by comparison with normal, i.e., control bladders.

Morphology of normal gall bladder epithelium: The mucosa of the gall bladder lumen consists of a continuous sheet of simple columnar epithelial cells. The layer rests upon a vascularized bed of loose connective tissue. The mucosa is thrown into frequent folds and convolutions according to the state of contraction or distension of the bladder as a whole. At the level of the light microscope, individual cells tend to be tall and columnar. Frequently, large intercellular spaces are observed between the lateral cell surfaces. Apically, however, the lateral spaces disappear and a slight, but not prominent, brush border forms the continuous surface of the epithelium. An oblong nucleus is generally visible near the middle of the cell. Occasionally, cytoplasmic vacuoles are noted (Fig. 6).
At the level of the electron microscope, the organelles in the ground substance of the typical epithelial cell are those common to cells in general. An elongate nucleus occupies a major portion of the cell volume. Characteristic are shallow indentations in its periphery and prominent invaginations of the nuclear envelope at its apical end (Fig. 7). Just inside the nuclear envelope, condensed chromatin appears. One or two nucleoli are usually present.

Portions of the Golgi are almost always seen in regions apical to the nucleus (Fig. 7). They appear in their usual array of closely layered cysternae flanked by small vesicles.

It is also common to find from one to four dark bodies, possible of lysosomal nature, in the region of the cytoplasm basal to the nucleus. Occasionally they are seen in the cytoplasm apical to the nucleus, and are conceivably a function of cell state (Fig. 7).

In both the apical and basal regions of the cytoplasm, mitochondria form populations so dense as frequently to appear contiguous. Normal mitochondria in cross section appear as oblong, ellipsoids (Fig. 7). The mitochondrial matrix is only slightly less dense than the surrounding cytoplasmic ground substance. From one to five granules
usually are seen randomly scattered in the mitochondrial matrix. Parallel bilaminar cristae extend with uniform spacing throughout the matrix.

Abundant vesicles of low electron density are normally found in the ground substance apical to the nucleus. While the very small vesicles usually appear devoid of internal substance, the larger vesicles commonly appear to enclose an amorphous substance that is more or less condensed (Fig. 7). These vesicles may be the source of extracellular substance found at the apical membrane (Ito and Revel 1964).

The most distal two or three microns of the apical cytoplasmic ground substance consists of a cortical zone normally exclusive of all organelles except for occasional vesicles (Fig. 7). Although the composition of the zone appears to be of a finely fibrin or felt-like quality, its overall density is very comparable to that of the remaining ground substance.

The cell surfaces of the mucosal epithelial cells are amplified to increase surface area in a fashion typical of transporting tissues (Fawcett 1962, Keynes 1969, Pease 1956, Philpott 1968). The basal surface is invaginated to form processes (Fig. 8) which are closely applied to a basal lamina. The basal lamina typically
appears as a fine fibrin-like network forming a continuous sheet (Fig. 8) beneath all the cells of the epithelium. The lamina is presumed to be freely permeable to fluids (Diamond and Bossert 1967).

The lateral surfaces of the individual epithelial cells constitute the walls of the intercellular spaces, or transport channels of Diamond (Figs. 7, 8) (Tormey and Diamond 1967, Diamond and Bossert 1967). Well defined intercellular spaces are always present between membrane processes of normal bladder epithelium (Figs. 7, 8). According to Tormey and Diamond (1967), the width of the spaces increases with increased transport activity. In the present study, however, considerable variation in the width of the spaces of control bladder epithelium were noted. These variations may simply be due to differences in section thickness or in physical conditions attending active transport and fixation. However, the question is raised as to the feasibility of indexing transport activity purely on the basis of the geometry of intercellular spaces.

At the apical end of the channel, the lateral membranes converge and run parallel for two to three microns before fusing to form a tight junction, or zonula occluden (Fig. 7). It is not uncommon at high resolution to find tight junctions with an apparent "space" between the outer
lamellae of opposed plasmalemmas (Inset Fig. 7). As will be demonstrated, this region is particularly susceptible to P.C. interaction.

The apical membranes of neighboring cells are brought into continuity at the junctional complex. Numerous microvilli are formed from the apical membrane of each cell (Fig. 7). The microvilli are not rigorously uniform in either size or distribution. In general, however, they are approximately 1.5 to 2.5 microns in length and about 0.5 microns in diameter. Spaces between microvilli may be larger than 1.0 micron. Thus, the surface of the mucosal epithelium is a continuous microvillar sheet (Fig. 7).

Finally, a surface associated material is found extending from the microvilli (Inset Fig. 7). The substance is filamentous in nature and generally resembles the antenuilae microvillares reported much earlier in the mouse gall bladder (Yamada 1955). The substance, at least in the tissue examined in this study, is not uniformly distributed among all the cells of the epithelium. That is, the amount, and perhaps the composition, of the surface substance may vary from cell to cell (Figs. 9, 10).
Morphology of Epithelium exposed to Polymeric Cations:
P.L. 175,000: The gall bladder mucosa epithelium was examined with both the light and electron microscope after the tissue had been exposed for 5 minutes and after it had been exposed for 30 minutes to each of the concentrations of P.L. tested,
a.) 0.1 ug/ml--At 0.1 ug/ml P.L. 175,000 in the bathing solution, no detectable morphological changes were found as compared to controls.
b.) 1.0 ug/ml--When the P.L. concentration was 1.0 ug/ml, the morphology at either the light or electron microscope level was not noticeably altered after a 5 minute exposure to the polymeric cation. However, after the 30 minute exposure, discontinuities in the mucosal epithelial sheet were apparent even with the light microscope (Fig. 11). This morphology is not characteristic of the epithelium as a whole, but represents one of the most obviously affected areas. When such areas were observed, they are invariably located at the crests of the mucosal folds as opposed to the crypts which appeared normal (Figs. 13, 14). It should be emphasized, however, that these alterations are real and characteristic of this experimental condition.

When the affected areas were examined with the electron
microscope, even more subtle changes in the cell ultra-
structure were apparent. The ground substance of some
cells appeared diluted out (Fig. 12). The microvillar
surface appeared to revert to more amorphous forms (Fig.
12). The region of the tight junctions becomes more
dense and contorted (Inset Fig. 12).

The membrane seemed to become pliable and flexible
and at the same time "sticky". That is, the membrane
appeared to lose its structural rigidity while demonstra-
ting a prevalent tendency to adhere to or fuse with juxta-
posed membranes (Fig. 12, Inset Fig. 12). For convenience
in further discussion, the term "lygeroglischrohymenosis"
(LGH) meaning "a state of the membrane being flexible
and sticky" is introduced here. As will be demonstrated,
evidence for such a membrane state is derived from repeated
observations of this type of structural change in membranes
exposed to polymeric cations. The changed structural
alteration is characterized by amorphous contortions and
convolutions of the membrane with a tendency of the mem-
brane to fold upon itself. These folds are apparently
the result of a fusion of the outer surfaces of the mem-
brane, the ultimate effect of which produces membrane
alterations in the form of tortuous sheets, multilaminar
vesicles, or dense "myelin-like" spirals (Figs. 12,15,16,18).
c.) 10.0 ug/ml--The same types of morphological alterations which were apparent at the level of the light microscope when the bladder is exposed to 1.0 ug/ml for 30 minutes are found when the tissue is exposed to 10.0 ug/ml. The difference is more quantitative than qualitative. The higher concentration causes alterations in more cells. The same conclusion is reached with respect to the difference between 5 minute and 30 minute exposures to 10.0 ug/ml P.L. as is illustrated by comparing Figures 13 and 14. After 30 minutes noticeably more cells are swollen or lysed, and debris is much more prevalent (Fig. 14).

Changes in the cell ultrastructure were much more common after 10.0 ug/ml P.L. than at 1.0 ug/ml, and myelin-like spirals were frequently observed (Fig. 15). Otherwise, the general types of alterations were the same as at the lower concentration. Again, the principal difference between the two exposure periods was quantitative. Swollen cells were observed apparently detached from the epithelial layer (Fig. 15). LGH figures were much more prominent in the region of the tight junction (Inset Fig. 15) where the juxtaposed membrane surfaces seem to adhere and form contorted sheets of double membrane. In the process of forming LGH figures, microvilli appear to be bent over, onto, or around each other, giving rise
to complicated folds, vesicles, and spirals (Fig. 16). Other microvilli seem to become shorter and stubbier (Fig. 17), and the apical surface in general contains considerably fewer microvilli than controls (Figs. 16, 17). Furthermore, the surface material seen in Figure 16 and to some extent in Figure 17, while rather apparent in its association with the free surface is not apparent when membranes fuse, i.e., it is either dissolved or completely condensed by the P.L. interactions, since there is no indication of a surface associated substance between fused membranes of the LGH figures (Fig. 18).

d). 100.0 ug/ml--The effects of 100.0 ug/ml P.L. after 5 minutes and 30 minutes were qualitatively the same as those described previously for lower concentrations. Quantitatively, however, the effects of 100.0 ug/ml after 5 minutes seemed to be somewhat less than the effects of 10.0 ug/ml after a 30 minute exposure (Figs. 14, 19). A 30 minute exposure to 100.0 ug/ml clearly caused more extensive damage to the epithelium than any other experimental condition (Fig. 20).

Examination of effected cells with the electron microscope clearly showed swollen and diluted cells. Abundant LGH figures were generally found in the apical region of the cell and were also associated with cell debris
(Fig. 21). Of perhaps more interest was the fact that the lateral surfaces of some cells were also clearly involved in LGH (Fig. 21). These membranes behaved in an analogous manner to those of the apical surface; i.e., the lateral processes became less defined, opposing membranes fused, and multilaminar figures formed. The lateral membranes of mucosa exposed to lower P.L. concentrations are observed to be involved in LGH figures, but to a lesser extent quantitatively.

P.L. 2,800: Generally, the effects of smaller molecular weight P.L. were remarkably similar to those of P.L. 175,000 (Figs. 21, 27). It did appear that P.L. 175,000 induced more cytological damage (Figs. 11, 14, 20) under identical experimental conditions than did P.L. 2,800 (Figs. 22, 25, 26).

a.) 0.1 ug/ml--Exposing the mucosa to this concentration of P.L. 2,800 for 30 minutes produced no change in morphology at the level of either the light microscope or electron microscope.

b.) 1.0 ug/ml--Examination of the tissue exposed to 1.0 ug/ml of P.L. 2,800 for 30 minutes with the light microscope showed only minimal alterations in the mucosa. Indications of slight discontinuities in the epithelium were occasionally observed (Fig. 20). The exposed epithe-
lium is seen to be in good condition as compared to controls (Fig. 22).

The ultrastructure of the epithelium corresponded well to good condition of cells as seen with the light microscope. However, a few cells were found with diluted ground substance and swollen nuclei and mitochondria but had not lysed. At the same time excellent examples of LGH formations could be found. Interestingly enough most of the LGH occurred in the region of the tight junction (Fig. 23), even though some involvement of the apical surface was occasionally observed. Furthermore, LGH could be demonstrated well within the lateral spaces (Fig. 24). However, myelin-like spirals and complex convolutions were generally lacking in the tissue treated under these experimental conditions.

c.) 10.0 ug/ml--The morphology at the level of the light microscope showed more generalized damage to the epithelial layer at 10.0 ug/ml than at 1.0 ug/ml (Fig. 25). Obvious ruptures in the mucosal layer were infrequent, but cytological damage was easily discernible as swollen, lightly stained cells, and as amorphous blebs along the mucosal surface (Fig. 25).

The ultrastructural integrity of the microvilli and lateral processes was obviously challenged. LGH was
clearly present.

d.) 100.0 ug/ml--After a 30 minute exposure to 100.0 ug/ml considerable swelling and lysis of cells, cell debris, and ruptures in the epithelium could be seen with the light microscope (Fig. 26).

It could be seen with the electron microscope that details of the architecture of both, the apical and lateral surfaces often were lost extensively (Fig. 27). LGH figures in the form of condensed myelin-like spirals, multi-laminar vesicles, and complicated membraneous infolding were common (Fig. 27 and Inset A, Fig. 27). Also, a thickening of the outer lamella of the trilaminar array of the plasmalemna was occasionally noted (Inset B, Fig. 27).

In general the effects of P.L. 2,800 on the ultrastructure were identical to the effects of P.L. 175,000. The larger molecular weight compound seemed to be somewhat more "powerful" in inducing LGH of the epithelial plasmalemna.

Protamine: The epithelial response to interaction with protamine was essentially the same as the responses elicited by P.L. However, at equal concentrations, protamine induced less structural alteration than either P.L. polymer.
a.) 1.0 ug/ml—No alterations in the mucosa were observed with the light or electron microscope when the tissue was exposed to 1.0 ug/ml of protamine for 30 minutes.

b.) 10.0 ug/ml—Exposure of the epithelial to 10.0 ug/ml protamine for 30 minutes produced breaks in the normally continuous mucosal layer and these were detectable with the light microscope (Fig. 28). A few lightly stained swollen cells were visible, but such alterations were restricted essentially to the prominent crests of the mucosal folds.

The most striking feature of the ultrastructure was the LGH of the tight junction regions. Although the apical membrane was occasionally disrupted and a loss of the microvillar form had occurred, the tight junctions showed LGH when neither of the former conditions were detectable (Fig. 25). High resolution showed that the membranes of the tight junction region were clearly fused and characteristically contorted (Inset Fig. 29). The resemblance of these structures to those produced by P.L. are quite obvious.

c.) 100.0 ug/ml—At the level of the light microscope cells were clearly lysed and/or swollen (Fig. 30). However, as remarked above, there was considerably less
debris and cell damage with this concentration of protamine than with similar concentrations of P.L. under the same conditions.

The ultrastructure of affected areas also revealed morphological changes quite similar to those described when the mucosa was exposed to P.L. Cells were lysed. Ground substance was diluted. Mitochondria were swollen. Nuclei were swollen and nuclear ground substance and chromatin were frequently condensed (Fig. 31). LGH was also present as multilaminar vesicles and adherent lateral membranes (Fig. 31, 32). Tight junctions appeared as contorted pentilaminar structures (Fig. 32). Microvilli were found which had apparently fused (Inset Fig. 32). And occasionally some thickening of the outer lamella of the tri-laminar plasmalemna was noted (Fig. 32).

Histone: Although histone caused mucosal damage almost proportionately to protamine at similar concentrations, it induced LGH only in rare circumstances. In this respect histone behaved quite differently than the three previously discussed P.C.

1.0 µg/ml—Like protamine, histone at a concentration of 1.0 µg/ml caused no detectable alterations in morphology at the levels of either the light or electron microscope.
10.0 ug/ml--At the level of the light microscope, morphological alterations induced by 10.0 ug/ml of histone were very similar to those caused by the same concentration of protamine. Breaks in the continuity of the epithelial layer due to lysed cells were occasionally observed, but again these were limited to the crests of mucosal folds.

The ultrastructure of cells exposed to this concentration of histone for 30 minutes showed surprisingly few alterations (Fig. 33). Amorphous blebs at the apical surface were seen, but not commonly. These alterations were associated with a loss of microvillar structure of condensed substance at the cell surface (Inset A Fig. 33). Of some note in contrast to protamine, the regions of the tight junctions were not obviously altered (Inset B Fig. 33).

100.0--The morphological damage to the mucosal epithelium as a result of exposure to 100.0 ug/ml histone is very comparable to that caused by other P.C. when viewed with the light microscope (Fig. 34). Numerous cells are swollen, diluted, and palely stained. There is some debris, presumably due to lysed cells. The continuity of the mucosal layer is clearly disrupted.

On the other hand, although cytological damage is
clearly present at the level of the electron microscope, there is essentially no LGH (Fig 35). The ground substance of some cells is obviously diluted. Mitochondria are clearly swollen. Swollen nuclei with condensed chromatin are seen. And the debris of lysed cells is prevalent. Fused membranes, multilaminar vesicles, and condensed "myelin-like" spirals are almost totally lacking, but they do exist. Rather than forming LGH figures, there seems to be a much greater tendancy of the membrane to vesiculate (Inset Fig. 35). Numerous vesicles of various sizes are noted in the cellular debris. Another distinction of the histone-membrane interaction is the frequent appearance of some precipitate or condensate usually associated with the apical surface and independent vesicles (Inset Fig. 35).

Morphology of Epithelium Exposed to Other Compounds:

Heparin--Mucosal epithelium exposed to 100.0 µg/ml of heparin for 30 minutes under the same physiological conditions as the P.C. showed no discernible morphological alterations with either the light or electron microscope.

Lysine amino acid--At a concentration of 100.0 µg/ml
lysine produced no detectable effect on the morphology at either level of examination.
Discussion

Chemical Purity of Polymeric Cations

The purity and molecular weight homogeneity of all polymeric cations used in this study were found acceptable by the assays performed. It is clear, however, that the arginine rich histone was a heterogeneous sample. For example, U.V. absorbance at 180 nm is a function of the peptide bond concentration in the absence of interfering factors (Schellman and Schellman 1964). Whereas equal concentrations (weight/volume) of both P.L. samples and the protamine sample demonstrated approximately equal adsorbances, the same concentrations of the histone sample showed significantly higher absorbances. This discrepancy is most likely due to the fact that there are a significant number of amino acid residues on the histone molecules which absorb in this U.V. region in addition to the peptide backbone. Furthermore, the tertiary conformation of the protein may alter its absorbing properties significantly (Schellman and Schellman 1964). Since histone is relatively heterogeneous with respect to amino acid composition (Murray 1964), it may possess structural properties distinct from those of the other samples.

In addition, disc electrophoresis revealed single
bands for P.L. 2,800 and protamine (P.L. 175,000 did not penetrate the separating gel significantly), but revealed several distinct bands of varying intensities for histone. It was therefore concluded that the histone sample consisted of several molecular species probably varying in molecular weight and charge density. However, the facts that the sample gave a positive fluoro-dinitro-benzene reaction and electrophoretically moved toward the anode, indicate that at least some of its components retain a net positive charge.

A word should be inserted regarding the fluoro-dinitro-benzene assay. This reaction was reported in the literature (Dubin 1960, Mamelak et al 1969) as an assay for P.L. In this study, however, the reaction consistently produced an insoluble precipitate from both molecular weight samples of P.L. The precipitate was insoluble in water, dioxane, ether, acetone, ethanol, propanol, and chloroform. On the other hand, the reaction products of protamine, histone, and L-lysine were soluble in dioxane. Hence, the assay in this study represents only a qualitative character of the samples tested.

Since two different molecular weights of P.L. were used to assay for effects due to molecular size, molecular weight parameters for each P.L. sample were examined.
Hence, for P.L. 175,000 a sedimentation coefficient of 0.98 S was determined, and by comparison to the data of Applequist and Doty (1962) and using the equation of Mandelkern and Flory (1952) a molecular weight of 150,000 characterized this sample. Even though this value is somewhat below that listed by the supplier, it is felt that the difference is insignificant for the present purposes. On the other hand, the consistent narrow peak of the schlerin patterns throughout the centrifuge run suggest good molecular weight homogeneity (Fig. 2).

Since P.L. 2,800 ran so rapidly during electrophoresis the possibility of low molecular weight contaminates was ruled out. Sephadex molecular sieving showed that the amount of low molecular weight contaminant was small since over 90% of the sample was recovered in the first void volume.

Relative Charge Densities of Polymeric Cations:
From what is known of the chemical composition of the samples and from the assays performed in this study, the following order of charge density is suggested for the polymeric cations studied:

P.L. 175,000 > P.L. 2,800 > Protamine > Histone

The epsilon amine group of each lysine in P.L. has a
pKₐ of 10.44 (Katchalski and Sela 1958) so that at physiological pH, most of these amines will bear a positive charge. Assuming that one terminal of each polymer bears an acid group ("COO⁻"), P.L. 175,000 should bear approximately 700 amine groups for each carboxyl, while P.L. 2,800 would contain only about 12 amine groups per terminal carboxyl. Protamine on the other hand is not a homopolymer, 70 - 80% of its residues bear positive charges at neutral pH (Mahler and Cordes 1966). Hence, the charge density of protamine should be somewhat lower than that of P.L. 2,800 and is reflected by the fact that it did run somewhat slower electrophoretically than P.L. 2,800. Histone, however, ran considerably slower than either P.L. 2,800 or protamine under electrophoresis. These results plus the fact that histone is known to have significant content of glutamate and aspartate residues which would reduce its net positive charge, leads to the conclusion that histone bears the smallest positive charge density of any of the compounds studied.
Effects of Polymeric Cations on Fluid Transport

At sufficient concentrations, all of the P.C. samples were inhibitory to fluid transport. However, the inhibitory effect of the P.C. per se offers very little information about the action of the P.C. At this point, it cannot be determined whether the effect is due to a reaction with the surface, to general toxicity effects on cell metabolism, or to specific inhibition of transport associated enzymes. With respect to the last possibility, a pilot project assaying the effect of 100 μg/ml P.L. 175,000 on the transport associated sodium potassium activated ATPase (Robinson 1970) was conducted*. Since approximately 50% of the enzyme activity as determined by a method similar to that of Fujita et al (1966) remained in the presence of this high P.L. concentration, it is doubtful that the inhibition is due totally to inhibition of this transport associated enzyme. However, in view of the data from other experiments indicating permeability changes, the active transport mechanism may be highly dependent on the specific selective permeability properties of the membrane. Needless to say, such speculations are fraught with difficulties, since, for instance, the effect of

*The assistance of Dr. S. A. Ernst is sincerely appreciated in assaying the enzyme activity.
mucosal ruptures on the total transport cannot be directly assayed.

Furthermore, it is noted that these transport studies show, in accordance with the morphological observations, a time dependent response to given P.C. concentrations (Fig. 3). That is, the fluid transport rate immediately after addition of a P.C. was consistently higher than the rate measured at some later time.

The principal value of these experiments was 1.) to demonstrate that P.C. do interact with the mucosal epithelium, and 2.) to indicate the concentration range at which the P.C. effectively alter transport.
Effect of Poly-L-Lysine 175,000 on Ion Perm-selectivity and Conductance Properties:

The effects of threshold concentrations (1.0-2.0 ug/ml) of P.L. 175,000 on the ion selective properties of the preparation were assayed in order to determine that the P.L. interaction was with the "membrane component" of the system. [It is felt that the P.C. interaction directly involves the surface material, defined previously as that substance exterior to the lipoidal phase of the membrane. However, since some confusion surrounds the term "membrane", the word, "membrane", is taken here to include all lipoidal and protein and/or protein-carbohydrate components and associated complexes.] Presumably, the perm-selective properties of the bladder preparation are essentially determined by the apical membrane of the mucosa (Diamond and Harrison 1966). Since the perm-selective properties of a membrane are a function of its fixed charged sites (Eisenman 1969, Scatchard 1955, Van Breeman 1965), alterations of the charge density of the membrane should alter the perm-selective properties of the membrane (Eisenman 1967, Diamond and Wright 1969). Furthermore, the permeability properties of a membrane may be adequately determined by such measurable parameters as transmembrane diffusion potentials (Eisenman 1967, Hodgkin and Katz 1949, Machen and Diamond 1970, and
Electrostatic interaction of P.L. with the membrane should lead to neutralization of negative sites. In turn, the charge reduction would be expected to produce perm-selectivity changes in the membrane. Therefore, diffusion potentials were measured before and after addition of P.L. to the bathing medium. The results (Table VI) clearly show a change in all diffusion potentials measured after P.L. was added to the mucosal solution. Furthermore, the changes in each case are toward the free solution junction potentials of the ions involved. The approximate junction potentials of the solutions considered are as follows:

\[
\begin{align*}
    K:Na &= 4.9\text{mV}, \text{K solution negative; Li:Na} = 2.7\text{ mV, Na solution negative; } 2:1\text{ Na} = 4.0, \text{ dilute solution negative.}
\end{align*}
\]

The above values are calculated with equations, solution conductivities, and ionic activities reported in the literature (Barry and Diamond 1970, Guggenheim 1930, Lyons 1967, MacInnes and Yeh 1921, Martin and Newton 1935, Robinson and Stokes 1968).

The ion permeability characteristics of the gall bladder for bi-ionic diffusion potentials studied here are followed by the Goldman constant field equation:
\[
V_m = \frac{RT}{F} \ln \frac{P_{Li} a_{Li}[Li]_m + P_K a_K [K]_m + P_{Cl} a_{Cl} [Cl]_m}{P_{Na} a_{Na} [Na]_s + P_K a_K [K]_s + P_{Cl} a_{Cl} [Cl]_s}
\]

(Goldman 1943, Hodgkin and Katz 1949). The transmembrane dilution diffusion potential, such as the 2:1 Na employed here, should be given by:

\[
V_m = P_{Na} a_{NaCl} - P_{Cl} a_{Cl} \cdot \frac{RT}{F} \ln \frac{a_{NaCl} (NaCl)_m}{a_{NaCl} (NaCl)_s}
\]

derived from the Henderson equation for diffusion potentials (MacInnes 1961). The symbols in the above equations are as follows: \(V_m\) = Transmembrane potential, \(R\) = gas constant, \(T\) = absolute temperature, \(P_x\) = permeability or mobility of specific ion in the membrane, \(a_x\) = individual ion or mean salt activity coefficient in solution, \((X)\) = concentration of ion or salt in solution where "x" is a specific ion or salt.

By inspection of the above equations, it may be seen that the value of the transmembrane potential, \(V_m\) in any case, is a function of the relative permeabilities of the ions concerned, holding other parameters constant. That is, the larger the differences between \(P_{Na}\) and \(P_{K}\), \(P_{Li}\), or \(P_{Cl}\), the larger the values of \(V_m\) will be. The essential point is that since \(P_x\) values depend upon the state of the membrane perm-selectivity, \(V_m\) may be taken as a reflection of that property. It is pointed out that the limiting
values of $V_m$ are the free solution junction potentials in which case the individual values of $P_x$ are the free solution mobilities of the ions considered.

Thus, it is now possible to say that changes in $V_m$ toward the free solution junction potentials after addition of P.L. reflect changes in the ratio, $\frac{P_x}{P_{Na}}$, toward its value in free solution. This condition results from a loss of membrane selectivity. That is, the ability of the membrane to discriminate between ions is decreased.

However, after examining the morphology of the mucosa exposed to P.L., it was clear that another interpretation of the reduced transmembrane potentials was possible. The morphology revealed that a small percentage of the cells had been lysed, creating holes in the continuous mucosal layer. Since the specific resistance of the intact membrane is many times that of an equivalent volume of salt solution, a discontinuity in the membrane would serve to short circuit the diffusion potential, $V_m$, produced by the remaining intact membrane, and the transmembrane voltage would clearly be reduced. In such a case it is possible to present the equivalent circuit,
where $V_m$ = transmembrane voltage produced by the diffusion potential across the intact membrane, $R_m$ = resistance of the intact membrane, $V_h$ = free solution junction potential across the "hole", $R_h$ = summed resistance of all holes in the membrane, and $V_t$ = total transmembrane potential seen by electrodes on each side of the membrane. $R_m$ and $V_m$ are dependent variables of membrane permeability, a change in which is reflected by $R_m$ and $V_m$ parameters simultaneously. It should be noted that the polarity of $V_m$ and $V_h$ will depend respectively on the sign of the membrane diffusion potential and the sign of the particular junction potential. In any case, with respect to $V_t$, the circuit is a voltage divider for both $V_m$ and $V_h$, the contributions of which to $V_t$ will depend on the relative values of $R_m$ and $R_h$. It is then possible to describe $V_t$ in terms of the following equation,

$$V_t = \frac{R_h}{R_h + R_m} (V_m) + \frac{R_m}{R_h + R_m} (V_h).$$

where the symbols retain the above definitions.

In equation 3 it is assumed that P.L. treatment changes $V_t$ by inducing changes in $R_h$, $R_m$, and $V_m$, which are regarded as dependent covariables of P.L. interaction. It is clear that $R_m$ and $V_m$ are entirely covariant, since
each is a parameter of the same phenomenon, permeability, of the same membrane. The nulling hypotheses suggested by the morphology is that \( R_h \) is the only variable in equation 3 affected by P.L. interaction. In this case, \( V_t \) is solely a dependent variable of \( R_h \). As defined above, \( R_h \) is the summed resistance of membrane holes and may be calculated from membrane conductance changes if \( R_m \) and \( V_m \) remain constant. If the nulling hypothesis holds, the calculated change in \( R_h \) should describe the observed changes in \( V_t \), and \( R_m \) and \( V_m \) are not affected by P.L.; i.e., the P.L. interaction induces no change in membrane permeability, but simply causes short circuiting of the diffusion potentials through holes in the membrane. On the other hand, if changes in \( R_h \) do not describe changes in \( V_t \), it is safe to continue with the assumption that \( R_m \) and \( V_m \) are altered and the permeability, affected by P.L. interaction.

The test of the null hypotheses ignores the decrease in \( R_m \) due to mechanical loss of membrane area in the formation of holes constituting \( R_h \). However, the decrease does not amount to more than a few percent, at most, and therefore, is insignificant in equation 3.

The task now becomes that of evaluating \( R_h \). From the equivalent circuit, it will be seen that
\[ R_t = \frac{R_h R_m}{R_h + R_m} \]

where \( R_h \) and \( R_m \) are as defined above and \( R_t \) is the total resistance across the membrane with "holes". Note that as \( R_h \) becomes very large (no "holes") \( R_t = R_m \), the resistance of the intact membrane before holes. Now, since all changes in the electrical parameters are by definition due to \( R_h \), it is only necessary to determine \( R_t \) after addition of P.L., which may be done by measuring and taking the inverse of the conductance, i.e., resistance. Likewise, since \( R_m \) remains constant, its value is obtained from the conductance measurement before addition of P.L. Equation (4) is solved for \( R_h \), and since all members of the right side of equation (3) are now known, a value of the transmembrane potential may be calculated. Clearly, if this value agrees with the observed values of transmembrane potential after P.L. addition, then the changes in diffusion potentials are likely to be due to the formation of short circuit "holes".

From the conductance results (Table VIII), \( R_m \) has an average value of 30.7 ohms·cm\(^2\). After exposure to P.L., \( R_t \) assumes a value of 24.9 ohms·cm\(^2\) and in order to satisfy equation (4) \( R_h \) is therefore assigned a value of 130 ohms·cm\(^2\). Inserting these values and the diffusion potential values, \( V_m \), from three experiments along with the appropriate
<table>
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<tr>
<th>Diffusion Potential Type</th>
<th>Expt. No.</th>
<th>$V_m$ original</th>
<th>$V_t$ observed</th>
<th>$V_t$ calculated</th>
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</thead>
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<tr>
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<td>7.2</td>
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<tr>
<td>&quot;</td>
<td>2</td>
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<td>&quot;</td>
<td>3</td>
<td>0.8</td>
<td>1.6</td>
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</tbody>
</table>

Table IX. Observed vs. Calculated Diffusion Potentials. The transepithelial diffusion potentials (3 types) in three experiments are given for $V_m$ original: the potential recorded before addition of P.L., $V_t$ observed: the potential actually recorded after addition of P.L., and $V_t$ calculated, the potential predicted by the equation of the equivalent circuit (see text).
junction potential, $V_h$, into equation (3), the hypothetical transmembrane potential, $V_t$ (calculated), is calculated and compared to the observed transmembrane potential, $V_t$ (observed) in Table IX. In each case, the observed potential is closer to the free solution junction potentials than predicted by equation (3). Therefore, parameters other than the formation of "holes" must be involved in reducing the diffusion potentials after addition of P.L. Since the change in conductance does not account for the decreased potentials, it is likely that the decreases are due to lower $V_m$'s from equation (1) which suggests again that there is a loss of membrane perm-selective properties.
Effects of Polymeric Cations of Morphology of Gall Bladder Epithelium

A survey of the morphological alterations due to P.C.-membrane interactions demonstrates the following: 1.) The order of effectiveness in inducing alterations corresponds to the proposed order of positive charge density. 2.) There are general similarities in structural changes of all the P.C.-membrane interactions examined, and 3.) There are some distinctions between the structural effects of the different P.C. investigated.

1.) Order of effect: At equal concentrations (1.0 ug/ml) P.L. 175,000 induced more general damage in terms of swollen, diluted, and lysed cells than did other P.C. under the same conditions. The difference between P.L. 175,000 and P.L. 2,800 seemed small, but the ultrastructure revealed that at low concentrations, P.L. 175,000 apparently induced more LGH figures in the apical surface than did P.L. 2,800.

At the same low concentrations (1.0 ug/ml) no alterations were observed with protamine or histone, but at 10.0 ug/ml changes were detectable. Under the light microscope, morphological changes were not noticeably different between 30 minute exposures to 10.0 ug/ml protamine and
the same concentration of histone (Figs. 28:, 39). However, ultrastructure changes showed that protamine produced considerably more LGH conformations than did the equivalent amount of histone (Figs. 29, 33).

2.) Similarities of Effect: Distinguishing between the morphological effects of the different P.C. with the light microscope is difficult without reference to threshold concentration and duration of exposures. The common alterations are characterized by some cells which are swollen, dilated, and lysed and by varying amounts of cytoplasmic debris associated with the free surface of the epithelial layer. Another feature common to these aberrations is that the affected cells are generally located at the crests of the folds in the mucosal layer as opposed to unaffected cells situated within the crypts of the folds. There are several possible explanations for this observation, all of which may play some role in producing the observed phenomena. a.) There may be a problem of diffusion of the P.C. into the narrow and restricted crypt areas such that cells at the crests are more exposed and vulnerable to P.C. than cells in the crypts. b.) There may be a higher concentration of extracellular substance within the crypts which inhibits diffusion to and/or interaction of the P.C.
with the surfaces of cells within the crypts. Candidates for such a substance might be residual secretory bile products not rinsed away or extracellular mucins (Ito and Revel 1964, Mercer 1964) accumulated at the cell surface and preserved within the crypts. The problem of distinguishing between the surface material composing the membrane and an extraneous surface coat is again apparent in this context. c.) There may be a significant difference between the chemical composition of the apical membrane of cells at the crests as compared to cells within the crypts (Figs. 13, 19). This possibility seems reasonable since the cells at the crests are "older" (Kaye et al 1966). Further to this point, a difference in the membrane composition between old and young red blood cells has been demonstrated (Marikovsky and Dannon 1969).

The similarities in P.C. interactions at the ultrastructure level are generally related to the morphological changes seen at the level of the light microscope. The underlying common features of the P.C. interactions are as follows:

a.) The apical membrane appears to become "leaky". Affected cells give the impression of having suffered osmotic shock. The ground substance is diluted. Mitochondria are swollen. Nuclei are swollen and nuclear
ground substance is diluted, and vesicles or vacuoles appear in a variety of states from swollen to ruptured (Figs. 21, 27, 31, 35). From these alterations it appears that the cell-surface loses its selectivity properties and significantly increases its permeability to extracellular solution. This conclusion is supported by the fact that the cytoplasm contains large molecules which cannot readily move through the membrane. If the membrane becomes significantly permeable to extracellular fluid, the fluid will enter the cell due to the osmotic gradient established by cytoplasmic macromolecules. The result is an increase in cell volume and diluted ground substance, both of which are observed. Since cells not exposed to P.C. do not show signs of swelling and cytoplasmic dilution, the permeability barrier of cells exposed to P.C. must be lowered as a result of the P.C.-membrane interaction. Needless to say, these points substantiate the above conclusions from diffusion potential and conductance changes that P.L. induced a nonselective increase in the membrane permeability.

b.) A loss of architectural integrity of the apical membrane is a common characteristic of all the P.C. interactions investigated. The structural rigidity of affected membranes seems diminished. Microvilli become
shorter and wider, lending a more amorphous appearance to the cell border. As the cell volume increases, the plasmalemma may be "stretched to the point that it has no remaining architectural integrity (Fig. 15). It could be argued that the loss of the structural integrity is simply due to the cell swelling, which undoubtedly has considerable influence on membrane form. It is believed however, from a survey of ultrastructure morphology that the loss of structure is at least concomitant to increased permeability, since in many instances the apical membrane appears altered before the cell shows detectable signs of swelling (Fig. 12, 15, 21).

c.) The term "lygeroglischrohymenosis" (LGH) introduced earlier, applies to a condition common to each of the P.C. interactions. The introduction of such a word (though not its length) may have some justification in view of the fact that previous reports have been made of the same general phenomena in other systems (Mamelak et al 1969, Katchalsky et al 1959, Sanders and Bell 1970). As the polymeric cation reacts with the cell surface, the membrane becomes flexible or plastic and tends to "stick" to other nearby membranes. High resolution electron microscopy shows that the "sticking" action involves a fusion of the outer lamellae of the trilaminar membrane structure
usually seen with osmium fixation. LGH figures always consist of, at least pentalaminar, and frequently multilaminar structures (Figs. 36, 37, 38 and Inset Fig. 32). It seems that if the membranes were joined without fusion of the outer lamellae, a heptalaminar structure should appear.

When multilaminar figures occur, three conditions potentially might arise. First, a definite space separates two or more pentalaminar membranes (Figs. 18, 36). In this case there is clearly no "fusion" of the "inner" lamellae. This pattern bears notable similarity to those reported previously for tight junctions (Farquar and Palade 1963) and synapses (Robertson 1963) as well as a number of other membrane forms of fusion (Elbers 1964). Second, the pattern of fusion may exist in an array almost identical to that observed in the Schwann cell of myelinated nerve (Robertson 1955). Here, the membrane appears "wound" very tightly with an alternating periodicity of major dense and minor dense lamellae separated by a constant intra-lamellar space as in the myelinate axon (Fig. 37). There may be some suggestion here as to a difference between the inner and outer lamellae as previously argued by Robertson (1955) and Wallach et al (1966). Third, the fusions may take place in such a way that there is no distinction
between the fused lamellae (Fig. 38). It is seen that such laminae are equally dense and symmetrical. The fact that there are apparent fusions of the "inner" lamellae in LGH formations (second and third conditions, Figs. 37, 38) is interesting since intuitively the P.C. is not expected to directly involve the "inner" surface of the membrane. It may be that the fusion of the inner lamellae is related only indirectly to the presence of a P.C. at the outer surface. That is, the fusion may result due to a change in transmembrane charge distribution, a change in geometrical proximity of whole membranes or components, or to a secondary change in membrane structure and chemistry.

Nonetheless, it is suggested here that the formation of these multilaminar figures may well be related to similar morphological figures that occur in nature. It has been accepted for quite some time that cell to cell junctions and adhesions are based in part on electrostatic bonds, forming an intercellular "bridge" (Ambrose 1964, Mercer 1964). It follows from the observations in this study, that a strongly basic protein within, or associated with, the surface material at points of cell to cell contact could perform this same "bridge"-type role. The report that a basic protein constitutes part of the protein fraction isolated from myelin preparations
(Wolfe 1962) seems relevant to these conclusions. Presumably, such a protein might react with the negative phospholipids as well as with other protein or carbohydrate associated anions.

Fourth and last, with respect to all P.C. examined, the molecular orientations of the different P.C. between the fused lamellae is likely to be similar. Using different molecular weights of P.L., Katchalsky et al (1959) observed that the distance between joined membranes was proportional to the chain length of the polymer forming the bridge. They suggested that the polymer was oriented normal to the plane of the juxtaposed membranes with one terminal attached to each membrane surface. P.L. 175,000 used in the present study should have an average length well over 1000 Å. In view of the fusion of the membranes observed here, Katchalsky binding mechanism seems hardly applicable. Rather, an orientation of the polymer such that the plane of its long axis is parallel to the plane of the fused membranes seems much more likely. Since the forms of LGH induced by the other P.C. are similar it seems probable, although not concretely demonstrable, that these polymers are oriented between the fused membranes in a similar fashion. Thus, as observed, the thickness of the fused membranes should be relatively independent of the
size of the "bridging" polymer.

3.) Disparities in the Effects of Polymeric Cations: Only very qualitative descriptions of the different responses elicited by the different P.C. are justifiable since each individual P.C. induces responses which vary considerably from cell to cell.

a.) P.L. 175,000--The distinguishing feature of the P.L. 175,000 membrane interactions seems primarily to be related to its size and possibly, also, to its higher charge density. At low concentration (1.0 ug/ml), P.L. 175,000 induces LGH configurations in the apical surface much more readily than other P.C. That is, microvilli across all of the apical surface of the cell, as well as tight junction regions, are affected (Fig. 12). It is plausible that these initial effects are due to the length of the polymer, just as Katchalsky suggested. Hypothetically, if the polymer were attached first by one end to the membrane surface, its ability to bind to a site on an adjacent membrane might well be a function of the length of the polymer. However, from this initial state of attachment, as additional sites on the polymer were reacted with the surface material, the membranes would be "pulled" together until the plane of the polymer was parallel to
the plane of the membranes. If this sequence of events is representative of the actual mechanism of LGH formation, it is not difficult to imagine that P.L. 175,000 might be more effective in fusing membranes which are spaced relatively far apart initially, e.g., the microvillar membranes versus the membranes approaching the tight junction.

The fact that P.L. 175,000 has a slightly higher charge density which may increase its interaction efficacy cannot be overlooked.

b.) P.L. 2,800 and Protamine--The general effects of P.L. 2,800 and protamine (aside from the concentrations at which they induced effects) were not noticeably different, and therefore will be considered together in this section. At their apparent threshold concentrations these two P.C., unlike P.L. 175,000, are limited primarily to the region of the tight junction as the site at which they induce LGH. In the control bladder a cross section in the region of the tight junction reveals opposing plasmalemmas that extend for 2-3 microns perfectly parallel to each other. A constant and easily discernible inter-membranar space is maintained. In addition the membranes of the junction fall along a straight line normal to the apical surface. However, contrasting this situation, the interaction of the P.C. occludes this inter-membranar
space, and the juxtaposed lamellae fuse (Fig. 23 and InsetFig. 29). Furthermore, the region of the junction does notremain straight, but becomes contorted and convoluted.

It is clear from the figure that the fused complex inducedby P.C. must involve several microns of plasmalemma beyondthe limits of the normal tight junction. The absence ofareas similar to normal junctions clearly indicates thatthe P.C. penetrated the junction. This observation mayhave some bearing on recent work calling into question the"tightness" of the tight junction (Friend and Gilula 1970,Martin 1970, Revel and Karnovsky 1967, van Lennep 1968).

Again, if the LGH forming capacity of a P.C. isinitially related to the polymer length, it would beexpected that these smaller polymers are most effectiveat the region of the tight junction where the juxtaposedmembranes are initially within "reach" of the polymer.

It follows that plasmalemma immediately adjacent to thetight junction would be fused as the membranes approachingthe seam of the tight junction are pulled within "reach"of the polymer by a "chain" reaction of membrane fusionextending away from the original tight junction.

P.L. 175,000 also affects the tight junction in a similarmanner but was not included here since it has the concomitant properties discussed above. Histone, as
noted below, seems not to affect the tight junction.

c.) Histone—The histone-surface material interaction exhibits several distinguishing features. One of the most noticeable features unique to histone exposure is the appearance of a considerable amount of floculent-like material associated with the apical surface and free vesicles (Inset Fig. 35). The substance appears to be the reaction product—a precipitate or condensate—of surface associated material with histone. However, it may only represent an adsorption of debris from lysed cells. Two observations discourage this conclusion. One, the material is seldom seen with other P.C. interactions even though cell debris is obviously present. Two, small quantities of the substance can be detected even when cell lysis is minimal; such is the case when low concentrations of histone were used (Inset A and Fig. 33).

A second distinguishing feature of the histone interaction is the presence of numerous free vesicles which occur in a variety of sizes. It appears that the microvilli and portions of the membrane have pinched off as blebs (Inset Fig. 35). Frequently, the floculent-like material mentioned above remains associated with such vesicles. The presence of vesicles is correlated with cell lysis, but lysis is not common at 10.0 ug/ml concentration.
The third unique feature of the histone interaction is the paucity of LGH figures even at 100.0 μg/ml concentration. Coils of fused membrane can be found, but they are not common. Instead, fragments of free "floating" membrane from lysed cells are observed frequently (Fig. 35). If the formation of LGH figures is a function of the charge density of the P.C., the scarcity of such figures in the presence of histone may be related to two considerations. One, histone may not be able to neutralize the surface negative charge effectively, and two, the low charge density dictates that greater concentrations of histone should be necessary for charge neutralization; thus, the sheer quantity (floculent-like material?) of histone that must be accumulated on the membrane may prohibit fusion.

Lastly, histone produces no detectable effects on the tight junction (Inset B, Fig. 33). This result is probably related to the factors discussed in the preceding paragraph.
Notes on Physical Chemistry of P.C. - Surface Material Interaction

Surface Adsorption: The relation between the amount of charged substance adsorbed to an electrostatically charged interface is defined by the following equation (Haydon and Taylor 1963, Rice and Nagasawa 1961).

\[ N_a = N_b \cdot e^{-\frac{zqU}{kT}} \cdot e^{-\frac{\Delta G}{kT}} \]

where \( n_a \) = amount adsorbed, \( N_b \) = concentration in the bulk solution, \( e \) = base of the natural log, \( z \) = valence of the adsorbed molecule, \( q \) = unit electron charge, \( U \) = surface potential in millivolts (see below), \( k \) = Boltzmann constant, \( T \) = absolute temperature, \( \Delta G \) = free energy interaction of the adsorbent with the interface. In the present system, if \( U \) is negative (\( z \) is positive) the first exponential should be positive. If the free energy of the interaction is essentially an electrostatic neutralization process, it should be negative and may have a value as high as 10 Kcal/mole (Pauling 1964); therefore, the second exponential should also be positive. If both exponentials are positive, adsorption should be a very favorable process and would be expected to follow a Langmuir isotherm (Giles and MacEwan 1957). Thus, even at very low concentrations in
the bulk solution, substantial quantities of the P.C. could be adsorbed to the surface material if the interaction satisfies equation (5). There is every indication that this is the case since P.I. at concentrations of only 1.0 ug/ml (about $5 \times 10^{-9}$M) induces detectable effects at the surface.

Surface Repulsion: The source of the repulsive forces between two juxtaposed membrane surfaces of like charge is their electrostatic surface potential, which for univalent electrolyte solutions is described by the Gouy equation (Dawson 1968),

$$ U = \frac{2kT}{q} \sinh^{-1} \frac{1}{AC^{1/2}} $$

where $A$ = area per charge, $C$ = total ionic concentration of the solution, and the other symbols retain the definitions above. $\sinh^{-1}$ is a log function so that $U$ is an exponent of a function describing $\frac{1}{A}$. It will be noted then, that $U$ increases as a function of decreasing $A$.

The actual force between two parallel membranes is described by Coulomb's law (Orear 1964),

$$ F = \frac{U_1 U_2}{Dr^2} $$
where $F = \text{electrostatic repulsive force}$, $U_1 = \text{net potential of one membrane interface described by equation (6)}$, $U_2 = \text{net potential of other membrane interface likewise described by equation (6)}$, $D = \text{dielectric constant of the medium}$, and $r = \text{distance between the membrane surfaces}$. Since $U$ is an inverse function of $A$, increasing $A$ will decrease the separating force between two membranes. Clearly, if $A$ increases during the P.C. interaction, the repulsive force between membranes will diminish. Furthermore, if at any moment $U_1$ attains a net charge opposite that of $U_2$, $F$ becomes an attractive force and the membranes would attract rather than repel. Hence, during the process of interaction, if an excess of P.C. is adsorbed to one of the parallel membranes an attraction, and fusion of the membranes would be anticipated. Nevo et al (1955) have clearly demonstrated that an excess of P.L. can be adsorbed to the red cell membrane.

Since P.C. are effective at low bulk solution concentrations with reference to equation (5) and since the P.C. induced fusion of membranes is reasonably well described by the considerations of equations (6) and (7), it is felt that the most probable mechanism of the P.C. - membrane interaction is that of an acid-base neutralization between the fixed negative sites of the membrane and the cationic sites of the polymeric cation.
Notes on the Physical Conditions of the Surface Material

From the observations on the effects of polymeric cations presented in this study and from observations by other investigators on the nature of the membrane surface, it seems both possible and appropriate to speculate as to the physical conditions attending the surface material. There are two assumptions necessary for this discussion, both of which have been defended previously. 1.) The surface of cell membranes bear a net negative charge. Although there is some evidence that the size of the surface potential may vary from one cell type to another (Cook 1967, Seaman and Pethica 1957), it seems that all cell surfaces are universally negatively charged (Mercer 1964). 2.) The negatively charged surface is due to fixed anionic groups which are characterized by electronegative centers, such as the resonating double bond oxygen. The most commonly sited source of the negative site is the carboxyl, more specifically in the form of sialic acid (Benedetti and Emmelot 1967, Cook 1968, Mercer 1964, Weiss 1970). Others include sulfate and phosphate esters. For the present argument, the specific radical seems unimportant since they are all characterized by strongly electronegative oxygens.

It can be shown that negatively charged interfaces
create electrostatic double layers in which the concentrations of counter ions are remarkably higher than in the bulk solution. The point in fact is that the pH of the interface may very several units from that of the bulk solution (Danielli 1936) and is related to the bulk pH by the following equation (Dawson 1968, Seaman and Pethica 1957, Weis 1970),

\[ \text{pH}_{\text{surface}} = \text{pH}_{\text{bulk}} + \frac{qU}{2.3 \ kT} \]

where all symbols retain the definitions given. Obviously, if \( U \) is negative, the surface pH will be lower, possibly by as much as 2 units, than the bulk pH (Seaman and Pethica 1957, Weiss 1963). There may be some reservation about the justification of equation (8) since the cell surface hardly represents a perfect interface (Stigter and Mysels 1955). The anionic sites are not located evenly at a plane of phase boundary, but are distributed throughout the matrix of the surface material (Cook et al 1961, Haydon 1964). In this case the ionic atmosphere is likely to follow a Donnan distribution (Hitchcock 1955, Passow 1969). However, this arrangement should not change the net result of a decrease in pH at or in the surface material as compared to the bulk solution. In fact, deviation from the Donnan equilibrium is in the direction of higher proton concen-
trations (Morawetz 1961). Hydrogen ions are preferentially adsorbed from the bulk solution frequently by a factor as great as 100, i.e. 2 pH units (McLaren 1957, Weiss 1970). Hydrogen Bonding: The surface material is characterized then, by groups which are highly electronegative, and by a substantial concentration of hydrogen ions. These two factors constitute the essential ingredients of and favor the formation of the hydrogen bond. In general, resonating oxygens of the form $\text{A}_2\text{O}_5^-$ and primary amines, $\text{R-NH}_3^+$ have sufficiently electronegative centers to form very stable H-bonds (Marsh et al 1955, Pauling and Corey 1954, Pauling 1960, Scherega 1963). The formation of intra-molecular H-bonds in structures such as the alpha helix configuration of proteins and DNA has been recognized for some time; however, the energies of these bonds are generally considered to be relatively low, usually less than 1-2 Kcal/mole (Applequist and Doty 1962, Katchalski et al 1964, Pauling and Corey 1954). Bonds of considerably higher energies with values as high as 9-10 Kcal/mole may be formed between carboxylic acid and amine groups, which have both an electrostatic and hydrogen bond character. Also, dimerization of acetic acid involves strong H-bonds of about 7.6 Kcal/mole (Pauling 1960). The exact value of the bond energy is a function of a number of parameters such as heat of neutrali-
zation, the dielectric atmosphere of the bond, and reduction of the degrees of freedom of the bonding sites (Astbury 1940, Pauling 1962). Over its range of values, the H-bond represents intermediate bond strength when compared to covalent bonds (50Kcal/mole or more) and Van der Waals-London bonds (a fraction of a Kcal/mole) (Moore 1964).

In addition, the H-bond is not highly specific. Its formation is determined almost entirely by the possible proximity (slightly less than 3 Å) and the strength of the two participating electronegative centers (Pauling 1962, Scheraga 1963). Exemplifying this situation, the H-bond may form as a pair between two acid groups

\[ R_1-C\equiv O\cdots H-O\cdots C\equiv R_2 \]

or it may form singularly

\[ R_1-C\equiv O\cdots H-O-R_2 \]

or it may form between an acid and base as in

\[ R_1-C\equiv O\cdots H-NH_2-R_2 \]

Fixed Anion Structure Theory: The conclusion from the above considerations and from the results of this study is that the fixed anionic sites are key structural elements of the membrane since they are capable of giving rise to stable hydrogen bonds within the surface material. (The role of such sites with respect to calcium has been emphasized previously (Singer and Tasaki 1968) and will be considered later here). The nature of the H-bond should make it particularly suitable to play a principal role in membrane stability. The formation of inter- and intra- molecular H-bonds in the surface material creates a continuous matrix that is responsible for membrane rigidity as well as permeability. The H-bond may be the basis of the protein polymerization suggested by Garret and Green (1970) to account for the structural stability of their model membrane.

Therefore the amount or degree of inter- and intra-molecular bonding determines the state of condensation or expansion of the matrix material complex. If there is little bonding, inter-molecular structure is lost and the matrix is expanded, not unlike forms of expanded polyelectrolytes in model systems (Katchalsky 1964, Michaeli 1960). If bonding is increased, inter-molecular structuring occurs and the matrix is condensed again
somewhat analogous to model polyelectrolytes. On this basis, increased bonding within the matrix is expected to produce heightened rigidity and decreased permeability. The permeability property follows since increased bonding and matrix condensation should lead to a decrease in the inner matrix volume, which if by nothing else restricts fluid movement by Poiselle's law (Moore 1964). It may well be that as the dimensions of the inner matrix volume approach inter-molecular distances, in which case Poiselle's law becomes inapplicable (Derjaguin 1963, Diamond and Bossert 1967, Kavanau 1965) and fluid movement would be even more restricted due to increased viscosities resulting from molecular ordering (Kavanau 1965).

If the above considerations hold, any action which affects the physical state of the fixed anion sites should produce corresponding changes in the physical condition of the membrane as a system. Any phenomena which leads to a significant loss or neutralization of fixed anionic sites predicts diminished membrane rigidity and increased permeability, since a.) the bonds associated with the affected anionic sites would be lost or disturbed, and b.) general hydrogen bonding would be decreased as a function of the hydrogen ion concentration which depends upon the net negativity of the matrix.
Any phenomena which lead to increased intra- and inter-
molecular bonding in the matrix is predicted to cause
increased structural rigidity of the membrane and de-
creased permeability by reversal of the above arguments.
Therefore it should be possible to predict general effects
on the membrane by adjusting parameters which principally
affect the anionic site, e.g., specific enzymatic digestion
of the sites, pH and calcium ion concentration, or effective
neutralization of the anionic sites with certain cations.

The following discussion is, therefore, an attempt
to coordinate the observations made in this study and
some of those made by other investigators with the hypo-
theses relating membrane structure and permeability to
the state of fixed anionic sites in the surface material.

Observations in the Present Study

a.) Effect of polymeric cations--The adsorbtion
of a cationic polymer to a negatively charged surface
reduces the interfacial electrostatic potential, U.
Likewise the interfacial pH is a function of U. P.C.
adsorbtion must therefore result in a decrease in the
hydrogen ion concentration at the cell surface. It
is noted further that adsorbtion of the P.C. may continue
beyond neutralization to produce a net positive inter-
facial potential (Nevo et al 1955). Under such circumstances the surface hydrogen ion concentration would be expected to decrease substantially below its normal concentration, perhaps by a factor as great as 10,000. Since H-bonding is influenced by the hydrogen ion concentration (Steinhart and Beychok 1964), adsorption of a P.C. should decrease intra- and inter-molecular H-bonding with a concomitant loss in structural rigidity and increase in permeability. Both predictions are verified by the experimental observations that the membrane conductance increases, there is a loss of perm-selectivity, and morphologically the membrane appears considerably less rigid and swollen cells indicate increase membrane permeability.

A note should be added regarding the molecular freedom introduced into the surface material matrix due simply to the interaction with a polymer such as P.L. The primary amine associated with the terminals of the polymer residue is expected to compete very favorably for fixed anionic sites such as carboxyls since the reaction has a relatively large enthalpy change. The resulting association therefore should be stable. However, the lysine amine occurs at the terminal of a saturated four carbon chain which allows it considerable freedom of
movement. Aside from increasing the bond stability by minimizing the entropy term in the reaction, this freedom of movement must also be imparted to the reacted fixed anionic site, which is expected to be revealed as a decrease in the total structural rigidity of the membrane as observed.

b.) Effect of calcium--A discussion of the experimental effects of calcium has been delayed until now so that it could be considered in the present context. Calcium inhibits the interaction of low concentrations of P.L. with the membrane. This effect of calcium may be explained at least in part, by assuming that increased calcium neutralizes fixed anionic sites thereby reducing U of equation (6), and hence by equation (5) the concentration of P.L. in the bulk solution necessary to produce the same amount of adsorption must increase. The observation that changing the concentration of P.L. from 1.0 ug/ml to 10.0 ug/ml overcomes the protective effect of calcium supports this conclusion.

On the other hand if this hypothesis is accepted, it is necessary to explain the reversibility of the calcium - membrane interaction since calcium should lower U, and the degree of H-bonding in the surface material.
Fortunately, it has been noted frequently that calcium and hydrogen ions mimic each other in their effects on films, polyelectrolytes, and biological membranes (Kavanau 1965, Michaeli 1960, Singer and Tasaki 1968, Steinhart and Beychok 1964). Although the basic mechanisms of binding for the hydrogen and calcium bonds are distinct, the fact that they exhibit similar effects may not be so surprising when it is recalled that each has properties of a double co-ordinate. This property gives each ion the common ability to form inter-molecular bridges. It has already been demonstrated that H-bonding may coordinate to acid groups, and needless to say, calcium could demonstrate the action between exactly the same two acid groups by electrostatic bonding. Hence, in this case substitution of calcium for H-bonding should lead to negligible conformation changes, since the electronegative centers will still be restrained by the di-valent salt bridge. It is understood, nonetheless, that the calcium bridge is neither equivalent to, nor expected to substitute for H-bonds in every case. Therefore, since the calcium-membrane interaction is reversible upon withdrawing the hypertonic calcium, it is assumed that the stabilizing effects of the di-valent bridge compensate for the loss of hydrogen bond stability. If, as hypothesized, the key role of the fixed anionic sites
is structural stability, the calcium effects are compatible.

In addition it is noted from results obtained here as well as from other studies (Wright and Diamond 1968), that calcium is capable of increasing the membrane stability or rigidity since the membrane conductance always decreases in the presence of elevated calcium concentrations.

Observations in Other Systems

a.) Enzymatic digestion--The removal of anionic sites from the surface material of the membrane is expected by the proposed theory to decrease the membrane structural rigidity. Enzymatic digestion with neuraminidase (Gottschalk 1958, Ravetto 1968) and with trypsin (Pigman and Gottschalk 1966, Price 1970) removes significant quantities of surface associated anionic sites (Cook et al 1961, Maddy 1969). Corresponding to the theoretical prediction, several investigators report loss of rigidity. Treatment with neuraminidase increased the deformability of Ehrlich sarcoma cells (Weiss 1965). Exposure of L-strain fibroblasts to trypsin significantly reduced the surface tension of these cells (Maddy 1969). Treatment of red blood cells with Sendai virus which apparently removes all traces of sialic acid, appears to
reduce the membrane's strength and increase its permeability (Howe and Morgan 1969). Sialidase treatment of embryonic chick muscle decreases the membrane rigidity (Kemp 1968). The report that the lack of sialic acid is responsible for the loss of excitability in the rabbit retina (Vanysek and Moster 1968) lends itself to a number of interpretations as well as the present suggestions.

b.) pH Changes--Increasing the pH of the bulk solution should lower the hydrogen ion activity in the surface material with the correlative effect of reducing intra- and inter-molecular bonds. Therefore, the present hypothesis predicts that ordered membrane structure should decrease and the permeability should increase with increased bulk pH. The hemolytic activity of basic pH on RBC's is well recognized (Glaeser and Mel 1964, Heard and Seaman 1961). It appears that the permeability of erythrocytes decreases with decreasing pH, although definite conclusions are eluded somewhat by haemolysis which occurs outside of a fairly narrow pH range (Theorell 1952). Increasing the pH of the bulk solution increases the resting conductivity of the squid axon (Rojos and Atwater 1968) and of the frog sartorius muscle (Hutter and Warner 1967). In addition, the squid axon
begins firing sporadically as if its membrane becomes "leaky" after exposure to basic pH (Singer and Tasaki 1968).

On the other hand decreasing the pH of the bulk solution should have the effects opposite to those of increased pH, namely, increased membrane rigidity and decreased permeability. Going from pH 7.4 to 2.4, the conductance of the gall bladder decreased by 52% (Wright and Diamond 1968). Acidic pH reduces the spike potential of the squid axon, presumably due to decreased membrane permeability (Singer and Tasaki 1968). Furthermore, surface film pressures have long been known to be sensitive to the bulk pH (Danielli 1936, Dawson 1968). Alexander (1942) found that acetimide hydrocarbon chains when condensed in monolayers over an acid solution exhibited marked rigidity which he ascribed to hydrogen bonding between the acetimide groups.

c.) Calcium—If the structural strength and integrity as well as the permeability properties depend largely upon the association and restriction of anionic sites in the surface material, increasing the calcium concentration should enhance stability and rigidity of the membrane by forming a di-valent electrostatic bridge.
between sites. Decreasing the calcium concentration should induce opposite effects. As mentioned above calcium frequently mimics the effects of corresponding changes in hydrogen ion concentration. Lack of calcium induces sporadic firing (Frankenhauser and Hodgkin 1957) and excessive calcium reduces the action potential of the squid axon (Singer and Tasaki 1968). Calcium free medium inhibits the association of sponge cells, causing them to round up (Humphreys 1965). Excess calcium decreases the permeability of the electrotonic junction between cells (Loewenstein 1967) and decreases the inulin permeability of toad bladder (Mannery 1966). At moderate concentrations, calcium stabilizes the RBC (Weinstein et al 1970), but at high concentrations results in hemolysis (Schoffeniels 1964). The action of calcium on artificial membranes generally decreases their permeability and conductivity (Gary-Bobo 1970, Palmer and Schmitt 1941, Shimojoi and Ohnishi 1967). Present in the bulk solution, calcium reduces the surface pressures and increases the collapse pressures of monolayer films, accordingly this action depends upon the negativity of the lipid polar groups (Leitch and Tobias 1964, Palmer and Schmitt 1941).

d.) Anionic Site Neutralization--Intra- and inter-
molecular H-bonding is expected to diminish in the presence
of such charge neutralizing agents as polymeric cations. In addition, the agents may effectively disrupt di-valent salt bridges between negative centers. Both actions are expected to reduce the membrane structure and increase the membrane permeability. The results of the present study have, of course, been reported and are in accord with these concepts. However, a number of other investigations have also produced similar and relevant results.

Membrane deformation in the RBC as a response to interaction with polylysine was among the first reports of such responses to charge neutralization (Katchalsky et al 1959). Blebbing of the plasmalemma of fibroblast (Kornguth et al 1961) and increased internal channel formation of the plasma membrane of amoeba (Sanders and Bell 1970) have been observed when P.C. are added in low concentration to the media of these cells. A loss of detail of the apical membrane of toad bladder exposed to P.L. is reported (Mamelak et al 1969).

Increased permeability of several membrane systems is suggested as a response to P.C. interaction. Hemolysis is a well known response to relatively high P.C. concentrations (Nevo et al 1955). Fibroblasts appear to grow leaky and lyse when exposed to more than 100 ug/ml polyornithine (Ryser and Hancock 1965) and polylysine
(Seljelid et al 1970). Beet cells treated with P.C. leak potassium (Osmond and Laties 1970) and beta cyanin (Siegel and Daly 1966). And from the leached appearance of cells, P.L. seems to increase the permeability of toad bladder epithelium (Mamelak et al 1969).

Artificial membranes and monolayer films are not without correlative responses to the presence of P.C. Few (1955, 1957) showed that interaction of a positively charged decapetide destabilized cephalin monolayers and increased the permeability of phospholipid spherules.

In view of this modest proposal, a few humble qualifications seem deserving, since there are, undoubtedly, examples of phenomena giving results quite contrary to that predicted by a fixed anionic site structure theory. Notably are the results obtained with the red blood cell which lyses when certain concentration levels of hydrogen or calcium ions are exceeded. It may be that these results are best accounted for by analogy with thin films. In systems employing negatively charged polar groups on lipids of thin films, increases in stability occur with increased addition of calcium up to a point at which the film begins to expand, and then it becomes increasingly less stable. The explanation of the phenomenon is merely that more
than one calcium ion per two sites is forced onto the membrane. The result clearly is that calcium no longer acts as a stabilizing bridge and the excess positive charge causes the lipid molecules to repel each other (Papahadjopoulos 1968, Tobias et al 1962). The same set of events with respect to increases in hydrogen ion concentrations are suggested to be at play in biological membranes.

Returning to the results with the RBC, increasing the calcium or hydrogen ion concentrations beyond a critical level may produce a net positive charge in the surface matrix causing inter-molecular repulsion and decreased stability possibly leading to lysis. The mechanism by which calcium may act has been considered. Hydrogen ions by protonation of certain groups such as secondary or tertiary amines would have the same effect of producing a repulsive intra-matrix charge leading to instability and lysis.

Contingent properties:

Although it is strongly suggested that the essential, if not primary, function of the fixed anions is to maintain or regulate the structure of the surface material matrix, it is understood that a number of properties are coincident or contingent upon the net negative surface
charge. For example, the surface charge represents an electrostatic potential energy barrier which may insure that membranes in close proximity do not fuse. One glance at the basal infoldings of the nasal salt gland (Martin 1970) or the apical membrane of the proximal convoluted tubule (Pease 1955) presents the question of what keeps these closely associated membranes separate. An electrostatic barrier seems logical enough as an answer.

It is also interesting that several presumably surface associated enzymes have optimal activities at acid pH. The fact that the pH of the membrane surface is lower than the bulk pH due to its negativity has been discussed. Examples of such enzymes are yeast invertase and chymotrypsin (Emmelot and Box 1970). However, other enzymes supposed to be associated with the plasmamembrane, such as Na\(^+\), K\(^+\)-ATPase (S. A. Ernst in preparation) exhibit neutral pH optima \textit{in vitro}. Whether the \textit{in vitro} activity represents an unnatural situation, the enzyme functions at a sub-optimal pH \textit{in vivo}, or the membrane is composed of a mosaic of higher and lower pH areas as suggested by Weiss (1970) cannot be determined here.
Effects of Heparin

On the basis of the fixed anion structure theory, heparin (a highly negatively charged bio-polymer) is not expected to interact with the membrane surface material since there is no reason to believe that it should affect the anionic sites of the surface matrix. In fact, if equation (5) holds for a heparin-membrane adsorption, both $z$ and $\Delta G$ are of a sign such that both exponentials of the equation remain negative and little if any adsorption is expected. Conveniently enough, there are no detectable signs of heparin interaction even at 100 ug/ml as determined in this study.

Effects of Lysin Amino Acid

On the basis of electrostatic properties only, it is difficult to explain the lack of effect of lysin amino acid at the relatively high concentration of 100.0 ug/ml. Lysine, having two positive amino groups and one negative carboxyl at neutral pH, bears a net unit positive charge.

Two considerations may explain to some degree why disruptive interaction similar to that of P.C. is not observed. First, the epsilon amine is not completely isolated from the alpha carboxyl. Thus, the positive ionic character of the amine should differ somewhat from that of
corresponding epsilon amines in P.L. or proteins. Furthermore, from simple inspection it is seen that of the P.C. examined, lysine singly has the lowest charge density, except, perhaps, for histone.

Second, lysine is not a polymer and therefore is not expected to be adsorbed to the membrane as are other P.C. Its concentration at the membrane should follow that of other small molecules and be determined by a Donnan distribution; thus, even if the molecule participates in disruptive interactions, it may not reach concentrations critical to membrane stability. Such a situation receives support by the suggestion that the interaction involves electrostatic neutralization and the distribution is Donnan, an equilibrium concentration must be reached before the membrane is neutralized completely, since the distribution depends on the net negative surface charge.

Whether this amino acid can and does act as a "di-valent" and/or "tri-valent bridge" is open to further speculation.
Concluding Comments

This study has attempted to examine and interpret the role of fixed anionic sites in the surface material of the cell membrane. This study, as well as previously reported observations, indicate that the fixed negative site is ideally suited to meet the structural requirements of the cell surface. The requirements for rigidity are satisfied through hydrogen bonding and di-valent cation bridges between electronegative centers. Such bonds are capable of creating a polymerized network—a matrix—of the surface material which strongly influences both, structural and permeability, properties of the membrane.

As an additional commentary, it is noted that considerable attention has been devoted toward linking the fixed negative site per se with specific ion selectivity and/or active electrolyte transport properties. It is suggested here that the role of the anionic site in such functions, if one exists, may be secondary to its structural role. For example, Berg et al (1965) and Passow (1969) believe the effective permeability barrier is "within" the membrane and determined by fixed positive charges. It is only too obvious that a material composed of elements characteristic of ion exchangers should show properties of an ion exchanger. Whether these properties "function"
in the cell is a question in itself. But to assume that they function simply because they exist is, of course, erroneous.
KEY TO ABBREVIATIONS IN TEXT

A - area per unit charge
Å - angstroms
a_x - activity coefficient of specific ion or salt, "x"
a.r.r. - average residual rate, average rate of fluid transport during an experiment period expressed as a percentage of the rate during the previous control period.
C - total ionic concentration
cm - centimeters
D - dielectric constant
e - base of natural logarithm
F - electrostatic force, or Faraday's constant
G - free energy of reaction
k - Boltzman's constant
Kcal - kilo calories
KV - kilovolts
K:Na - experimental arrangement placing a potassium chloride solution on the mucosal side of a membrane preparation with an iso-osmotic sodium chloride solution on the mucosal side
LGH - lygeroglyschohymenosis, state of flexible adhesive membranes
Li:Na - identical to K:Na, except potassium is replaced by lithium
ml - milliliter
\textit{mu} - millimicrons

\textit{mV} - millivolts

\(N_a\) - amount adsorbed to a surface

\(N_B\) - concentration in the bulk solution

\textit{nm} - nanometers

\textit{O.D.} - outside diameter

\(p\) - probability value determined from student's \(t\) test

\(P_x\) - permeability of specific ion, "x"

\textit{P.C.} - polymeric cations

\textit{P.L.} - poly-L-lysine

\textit{P.L.} 2,800 - poly-L-lysine, molecular weight 2,800

\textit{P.L.} 175,000 - poly-L-lysine, molecular weight 175,000

\(q\) - unit electron charge

\(R\) - gas constant

\(r\) - distance between two surfaces

\(R_h\) - summed resistance of all holes through the epithelial layer

\(R_m\) - resistance of the intact epithelial layer, exclusive of holes

\(R_t\) - total resistance across the membrane preparation

\textit{S} - sedimentation coefficient

\textit{T} - absolute temperature

2:1 \textit{Na} - experimental arrangement placing iso-osmotic solutions on each side of the membrane preparation with the solution on the serosal side containing twice the concentration of sodium chloride as that on the mucosal side
$U$ - surface potential at a boundary

$ug$ - microgram

$V_h$ - free solution junction potential

$V_m$ - potential difference across the intact epithelial layer due to diffusion potential

$V_t$ - total potential difference across the membrane preparation

$z$ - valence of ionic or molecular specie
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Figure 1. Arrangement for Measuring Gall Bladder Diffusion Potentials

"X" Cl is any one of the physiological solutions: Li, K, or 1/2 Na, listed in Table I.
Figure 2. Schlierin Patterns of Poly-L-Lysine Boundary in the Analytical Centrifuge. Patterns photographed from top to bottom at 0 minutes, 32 minutes, and 76 minutes after reaching speed.
Figure 3a. Effect of Increasing Concentrations of Polymeric Cations on Active Fluid Transport
At 15 minute intervals, the concentration (ug/ml) of P.C. in the mucosal solution was increased by 10 fold as indicated. The total weight of fluid transported is plotted against time. The slope during each interval is proportional to transport rate. No P.C. was present in the solutions during the first 15 minute interval. Ordinate: milligrams fluid transported. Abscissa: minutes time. See Fig. 3b for control.
Figure 3b. Effect of Lysine Amino Acid and Heparin on Active Fluid Transport
After 15 minutes 100 μg/ml of Lysine and heparin were added to the mucosal solution of an in vitro transporting bladder. Total amount of fluid transported is plotted versus time. No compounds were added to the mucosal solution of the control or to the experimentals during the first 15 minute interval. Ordinate: milligrams fluid transported, Abscissa: minutes time.
Figure 4. Inhibitory Effect of Different Concentrations of Poly-L-Lysine on Fluid Transport

After 30 minutes of fluid transport in Bicarbonate Ringer’s, the following concentrations of P.L. were added to the mucosal solution of each group of experiments plotted above as indicated by the arrows: (A) control - no P.L. added, (B) 0.1 ug/ml, (C) 1.0 ug/ml, (D) 10 ug/ml, (E) 100 ug/ml. Slopes obtained by least mean squares. Ordinate: total milligrams fluid transported. Abscissa: minutes.
Figure 5. Effects of 10x Calcium plus P.L. 175,000 and P.L. 175,000 in Na-Ringer's.
Arrows indicate time of change of mucosal solution to that indicated. Dots indicate value of conductance at corresponding time. Ordinate: conductance in ohms cm$^{-1}$ x 10$^{-3}$. Abscissa: minutes.
Figure 6. Normal Gall Bladder Epithelium
Tissue exposed to bicarbonate Ringer's solution for 30 minutes at 37° C. Note similar appearance of mucosal cells (mc) and continuity of epithelial layer (el). The mucosa rest upon a continuous bed of loose connective tissue (lc). Magnification: 600 diameters.
Figure 7. Normal Gall Bladder Epithelium
Note equal densities of mitochondria (M), nuclei (N), and ground substance as well as the uniformity and detail of microvilli (mv) and lateral processes (lp). Tight junctions (tj) are uniform and straight. The cortical zone (cz) is constant from cell to cell and virtually exclusive of organelles. Inset: Note apparent space between plasma-membranes of the junctional complex (tj), the uniformity of the microvilli (mv), and presence of surface material (sm) at the tips of microvilli. Tissue was exposed to the same conditions as in Fig. 6. Magnification: 4,400 diameters; Inset, 18,000 diameters.
Figure 8. Basal Region of Normal Gall Bladder Epithelium
The basal lamina (bl) is a continuous layer separating mucosal cells (MC) from loose connective tissue (LC). Note that although the intercellular space is almost closed, the membranes of the lateral processes (lp) give no indication of fusing or adhering to each other. Tissue was exposed to the same conditions as in Fig. 6. Magnification: 8,000 diameters.
Figure 9. Normal Apical Surface of the Mucosa
Note very little surface associated material can be seen affiliated with the exterior of the plasmalemma. Magnification: 10,000 diameters.
Figure 10. Normal Apical Surface of the Mucosa
The presence of surface associated material (arrows) is demonstrated exterior to the apical membrane. Magnification: 11,000 diameters.
Figure 11. Gall Bladder Mucosa Exposed to 1.0 ug/ml P.L. 175,000 for 30 Minutes
Interruptions in the normally continuous epithelial layer is demonstrated. Magnification: 500 diameters.
Figure 12. Mucosal Cells Exposed to 1.0 μg/ml P.L. 175,000 for 30 Minutes

The architectural detail of the microvilli (mv) is compromised. The ground substance of some cells (D) is diluted. "Lygeroglischrohymenosis" (lgh) is demonstrated affecting tight junction regions as well as the apical surface (Inset). Magnification: 5,000 diameters; Inset, 22,000 diameters.
Figure 13. Epithelium Exposed to 10.0 ug/ml P.L. 175,000 for 5 Minutes
Rupture of the mucosal layer is apparent which is accompanied by cell debris and swollen palely stained cells. Note cells within crypt (cp) appear unaffected. Magnification: 575 diameters.
Figure 14. Mucosa Exposed to 10.0 ug/ml P.L. 175,000 for 30 Minutes
Swollen cells, debris, and rupture of the mucosa are obvious. Cells within crypts (cp) appear normal. Magnification: 525 diameters.
Figure 15. Epithelial Cells exposed to 10 ug/ml P.L. 175,000 for 5 Minutes. Coils of condensed membrane (lgh) are prevalent along the apical border. Contortion and convolution of the tight junctions (Inset tj) are common. Note the swollen cell (SC) shows every indication of osmotic trauma. Magnification: 6,000 diameters; Inset, 12,000 diameters.
Figure 16. Apical Surface Exposed to 10.0 ug/ml P.L.
175,000 for 5 Minutes
Following the patterns of the convoluted surface membranes indicates how microvilli and areas of the cell surface fold in relation to each other to give rise to tightly wound spirals and other LGH forms. Note the presence of surface associated on free surfaces, but not within fused membranes. Magnification: 20,000 diameters.
Figure 17. **Apical Surface Exposed to 10.0 ug/ml P.L. 175,000 for 3 Minutes**

Structural detail of Microvilli (mv) is lost. Microvilli become stubbier, less uniform, and generally more amorphous. Magnification: 20,000 diameters.
Figure 18. Apical Surface Exposed to 10.0 ug/ml P.L. 175,000 for 30 Minutes
Fused membranes appearing as pentalaminar configurations are demonstrated. Note symmetry of dense laminae (arrows) and absence of surface associated materials within LGH forms. Magnification: 37,000 diameters.
Figure 19. Epithelium Exposed to 100 ug/ml P.L. 175,000 for 5 Minutes
Alterations are very comparable to those in Fig. 8. Note cells within crypts (cp) appear normal. Magnification: 600 diameters.
Figure 20. Epithelium Exposed to 100 ug/ml P.L. 175,000 for 30 Minutes
Cell damage and debris were more widespread due to the above conditions than to any other examined. Magnification: 550 diameters.
Figure 21. Mucosa Exposed to 100 ug/ml P.L. 175,000 for 30 Minutes
Note osmotically traumatized cells (D), cell debris, and LGH figures (lgh). Surface membranes of the lateral space are fused (asterisks). Magnification: 6,500 diameters.
Figure 22. **Gall Bladder Epithelium exposed to 1.0 ug/ml**

P.L. 2,800

General structural alteration of mucosa is minimal, although close examination shows some damaged cells (arrowheads). Magnification: 475 diameters.
Figure 23. Apical Surface Exposed to 1.0 ug/ml P.L. 2,800 Junctional complex is contorted and abnormally dense due to fusion of the juxtaposed plasmalemmae. Magnification: 15,000 diameters.
Figure 24. Intercellular Space of Mucosa Exposed to 1.0 ug/ml P.L. 2,800. Fusion of the membranes of the lateral processes is clearly demonstrated. Magnification: 15,000 diameters.
Figure 25. Epithelium Exposed to 10.0 ug/ml P.L. 2,800
Palely stained cells and cytoplasmic blebs indicate mucosal trauma. Magnification: 600 diameters.
Figure 26. Epithelium Exposed to 100 \(\mu g/ml\) P.L. 2,800
Damage to the mucosa is clearly more widespread at this
higher concentration of P.L. Magnification: 575 diameters.
Figure 27. Mucosal Cells Exposed to 100 μg/ml P.I. 2,800 Abundant LGH forms and swollen cells (D) occur. Inset A: The complexity of LGH wound spirals and vesicles is demonstrated. Inset B: Thickening of the outer lamella of the plasma membrane is sometimes observed (arrow). Magnification: 5,000 diameters; Inset A, 35,000 diameters; Inset B, 54,000 diameters.
Figure 28. Effects of 10 ug/ml Protamine on Gall Bladder Mucosa
Magnification: 550 diameters.
Figure 29. Mucosal Cells Exposed to 10.0 ug/ml of Protamine
Note consistent involvement of tight junction regions (tj) and typical LGH contortion of the tight junction (Inset tj). Magnification: 4,500 diameters; Inset, 35,000 diameters.
Figure 30. **Effect of 100 ug/ml of Protamine on Mucosa Layer**

Magnification: 500 diameters.
Figure 31. Epithelial Cells Exposed to 100 μg/ml Protamine
Swollen nuclei, swollen mitochondria, and diluted ground
substance indicate osmotic trauma. Note multilaminar
vesicles and fused membranes of the lateral processes.
Nuclear ground substance is condensed in affected cells.
Magnification: 4,000 diameters.
Figure 32. Mucosal Cells Exposed to 100 ug/ml Protamine
Note complex conformation of tight junction regions (tj)
extending into the lateral spaces (asterisks). Inset:
Note symmetry of pentalaminar membranes (arrow) and thick-
ening of outer lamellae of plasmalemma (arrow). Magnifi-
cation: 11,000 diameters; Inset, 28,000 diameters.
Figure 33. Mucosal Cells Exposed to 10.0 ug/ml Histone
General Morphology remains fairly well preserved. Loss of
uniform microvillar structure at the apical surface is
noted (ap). Less dense appearing cells (D) indicate
cytoplasmic dilution. Inset A: Substance is condensed
at the exterior of the apical membrane. Inset B: Tight
junction is normal with less dense space between the
opposed membranes. Magnification: 4,800 diameters; Inset
A, 10,000 diameters; Inset B, 54,000 diameters.
Figure 33. Mucosal Cells Exposed to 10.0 μg/ml Histone
General Morphology remains fairly well preserved. Loss of
uniform microvillar structure at the apical surface is
noted (ap). Less dense appearing cells (D) indicate
cytoplasmic dilution. Inset A: Substance is condensed
at the exterior of the apical membrane. Inset B: Tight
junction is normal with less dense space between the
opposed membranes. Magnification: 4,800 diameters; Inset
A, 10,000 diameters; Inset B, 54,000 diameters.
Figure 34. **Effect of 100 ug/ml Histone on the Mucosal Layer**

Cellular alterations appear similar to those produced by high concentrations of other poly cations. **Magnification:** 525 diameters.
Figure 35. Epithelium Exposed to 100 µg/ml Histone
General appearance is that of osmotically traumatized tissue. Note lack of LGH forms; membranes tend to be "free floating" or vesiculated (Inset). Note also presence of condensate associated with the apical surfaces and membrane fragments (arrows). Magnification: 2,700 diameters; Inset, 3,800 diameters.
Figure 36. Pentalaminar LGH Figures
Fused membranes are separated by large spaces. Magnification: 45,000 diameters.
Figure 37. *Tightly Wound LGH Figures*
Fused membranes from dense lamellae of alternating densities. Magnification: 125,000 diameters.
Figure 38. Uniform Multilaminar LGH Figures
There is no distinction between dense lamellae of the fused membranes. Magnification: 125,000 diameters.
Figure 39. Effects of 10.0 μg/ml Histone on the Mucosal Layer
Magnification: 600 diameters.