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Physiological and Morphological Effects of Poly-*L*-Lysine on the Toad Bladder

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Summary. Studies were carried out on the morphological and physiological effects of the binding of poly-L-lysine (polylysine; mol wt $\cong$ 120,000) to the apical surface membrane of the toad bladder epithelium. Paired hemibladders were mounted in chambers and exposed to polylysine concentrations of 2, 8, or 80 μg/ml in the mucosal medium for periods of up to 2 hr. Radioautographs prepared after addition of ^{3}H-polylysine showed that the polymer was localized to the apical surface of the epithelium and in dense subapical masses in lysed cells. No significant morphological changes were seen in the epithelium by light or electron microscopy at polymer concentrations of 2 and 8 μg/ml. Exposure to 80 μg/ml lysed many epithelial cells, i.e., converted them to slightly swollen ghosts with pycnotic nuclei and empty cytoplasm, except for remnants of mitochondria and vesicular fragments of the endoplasmic reticulum. All of the superficial epithelial cells were lysed in stretched hemibladders. The plasma membranes of the lysed cells were uniformly thickened, and their intercellular attachments remained intact. In contracted hemibladders, lysed and normal-appearing cells were interspersed, and the number of lysed cells in the epithelium was proportional to the duration of exposure to high concentrations of the polycation. In parallel experiments, the effects of varying concentrations of polylysine on active Na^+ transport and osmotic flow of water were measured with and without vasopressin, aldosterone, or amphotericin B in the media. At a concentration of 2 μg/ml of polylysine in the mucosal bathing solutions, no change in the basal rate of Na^+ transport was seen, and the response to vasopressin was unimpaired. At a concentration of 8 μg/ml, there was a significant but small fall in electrical potential difference (PD) and in short-circuit current (SCC) and no interference with the response to vasopressin. At a concentration of 80 μg/ml, there was a rapid curvilinear fall in SCC to $54 \pm 4\%$ of the baseline value and in PD to $21 \pm 3\%$ of the baseline value in a 2-hr period. Simultaneous unidirectional isotope flux studies with ^{22}Na and ^{24}Na showed a more than twofold increase in the serosal to mucosal flux but no discrepancy between net flux and SCC. Despite the inhibitory action of the polymer, the stimulatory response in Na^+ transport to vasopressin, aldosterone, and amphotericin B was relatively preserved in that the percentage increase in SCC was the same in the polymer-treated and control hemibladders. The polycation produced a small but significant increase in osmotic water flow, and striking and irreversible inhibition of the water-flow response to vasopressin.

In previous studies, distinct roles have been assigned to the apical and basal-lateral plasma membranes in transepithelial transport of salt and water. In the Koefoed-Johnsen-Ussing model [20], the apical (lumenal or

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outward-facing) surface of the cell membrane is designated as a selective permeability barrier, and the basal-lateral (serosal or inward-facing) surface is designated as the site of the Na^+ pump. These boundaries (apical and basal-lateral cell membranes) have also been assigned separate roles in the mechanisms of action of a variety of hormones and drugs [10, 15, 23, 33, 36]. If the permeability properties of one or the other surface of the epithelial cell membrane could be altered selectively, further information might be obtained on the nature of the function served by each boundary in the transport process. The substances used to modify membrane permeability should bind only to the exposed surface membranes and be excluded from the interior of the cell in order to insure that the effects do not directly involve other components of the transport machinery.

From earlier studies, we inferred that cationic poly α-amino acids would interact strongly with epithelial cell membranes and produce profound changes in their permeability and selectivity properties. Lipman, Dodelson, and Hays [24] deduced from electrophoretic and Ca^{++}-binding experiments that toad bladder epithelial cells carry a net negative surface charge at neutral pH. Poly-L-lysine (polylysine), a polycation with one positive charge per lysine residue at neutral pH, binds strongly to red cell and ascites tumor cell membranes at negatively charged sites [17, 21]. Moreover, the binding process is reversed on exposure of the surface to polyanions, and the polycation appears to be excluded from the interior of the cell [27]. These findings prompted us to study the physiological and morphological effects of exposing the mucosal surface of the urinary bladder of the toad to high molecular weight polylysine.

Methods and Procedures

The South American variety of *Bufo marinus* was used in all of our studies. The animals were kept on moist bedding at room temperature prior to use, and all experimental procedures were done at 22 to 24 °C unless specified otherwise.

Transport Experiments

Short-Circuit Current (SCC) and Potential Differences (PD). The urinary bladders were excised after double pithing of the toads, and each hemibladder was mounted in a glass chamber and incubated overnight in frog-Ringer's solution fortified with glucose (10^{-2} M), penicillin G (0.1 mg/ml), and streptomycin (0.1 mg/ml) as described previously [30]. The following morning, all solutions were replaced with fresh frog-Ringer's solution containing glucose (6×10^{-3} M). The time of addition of the polymer or of hormone was designated as time zero. Active Na^+ transport was estimated by the SCC method of Ussing and Zerahn [37] and expressed as μamp/2.54 cm^2 of exposed bladder. In some studies, the bladders were exposed to the polymer after only 2 to 4 hr of preincubation. The details of the protocols are given with the results.

Water Flow Experiments. Rates of water flow across hemibladders along fixed osmotic gradients were measured in glass chambers with orifice cross sections of 7 cm^2 as described previously [11]. The serosal bathing medium was frog-Ringer's solution (osmolality, 0.235), and the mucosal bathing medium was frog-Ringer's solution diluted 1:10 with distilled water (osmolality, 0.0235). In all experiments, pretreatment flow rates were measured for 0.5 hr, and hemibladders with flow rates greater than 1 μliter/min were discarded. The details of the experimental protocols are given with the results.

Radiosodium Flux Experiments

The simultaneous bidirectional flux of Na^+ was estimated with two radioisotopes. $^{22}NaCl$ (0.3 μc/ml, final concentration) was added to the serosal solution and $^{24}NaCl$ (1.5 μc/ml, final concentration) to the mucosal solution, and 0.5-ml aliquots of the bathing media were withdrawn at 30-min intervals. The samples were assayed for ^{22}Na and ^{24}Na by differential counting in a two-channel autogamma system (Packard Instrument Co.), both before and after total decay of ^{24}Na (i.e., 33 half-lives). The count rates were corrected for background and radioactive decay. All flux values were calculated in units of μamp/2.54 cm^2 of exposed surface and were then normalized by dividing each value by the mean SCC measured in the 1.0-hr period prior to the addition of polylysine. This procedure simplified the calculations.

Radioautography

Poly-L-lysine hydrogen bromide was tritiated by the Wilzbach method at the New England Nuclear Corp. The radioactive product was purified by chromatography in a 45 × 2.5-cm column of 200 to 400 mesh Bio-Gel A-5 m. The sample was eluted with 1.0 M NaCl, 2.4 mM $NaHCO_3$ (pH 7.5) buffer at room temperature. The column was calibrated with polylysine of mol wt 42,000 and 140,000. The peak centered at 140,000 mol wt was used for radioautographic studies. This material was dialyzed exhaustively against frog-Ringer's solution at 0 °C and diluted to 80 μg/ml by addition of frog-Ringer's solution. The polylysine concentration in these solutions was measured by the change in optical density at 225 mμ and by the method of Dubin [8]. The ^3H-polylysine frog-Ringer's solution was exchanged for the mucosal bathing medium at time zero. In one set of experiments, four hemibladders in either a stretched or contracted state were exposed for 2 hr to the mucosal ^3H-polylysine solution. The contracted hemibladder was mounted in glass chambers as described previously [9]. The remaining three hemibladders were stretched to various degrees over a plastic ring 3 cm in diameter which was then inserted as a diaphragm between the flanges of the glass chambers. In a second set of experiments, the duration of exposure to ^3H-polylysine was varied. The details of the protocols are given with the results.

At the end of the experimental periods, the mucosal solution was replaced with 0.25 M sucrose for 10 min to remove unbound or loosely bound polylysine. The hemibladders were removed from the chambers and fixed in 1% osmium tetroxide buffered with veronal acetate (pH 7.5) in 1% sucrose [3, 28], dehydrated in ethyl alcohol, and embedded in araldite or epon [25]. Sections 0.5 to 2.0 μ in thickness were mounted on glass slides and coated with diluted Kodak NTB-2, NTB-3, or Ilford L-4 emulsion by the method of Messier and Leblond [26]. The sections were exposed for 0.5 to 8 months, developed in Kodak D-19 or Microdol X developer, and fixed in Kodak acid fixer. Some of the sections were poststained with Kingsley stain [1] or 1% toluidine blue in 1% borax [35].

Electron Microscopy

The hemibladders used for electron microscopy were mounted on plastic rings, incubated, and harvested as described in the section on radioautography. After fixation in 1% osmium tetroxide, small blocks, 1 mm on a side, were cut, dehydrated in ethyl alcohol, and embedded

in epon. The blocks were stained with uranyl acetate during the embedding procedure [13, 18]. For light-microscopic examination, sections 0.45 μ thick were cut and stained with 1% toluidine blue in 1% borax. For electron-microscopic examination, sections 600 to 800 A thick were cut and were stained sequentially in 5% uranyl acetate at 37 or 60 °C for 30 to 45 min and in lead citrate [38] for 15 min. The latter step was carried out in a humid oxygen atmosphere. The thin sections were examined in a Siemens Elmiskop I operated at an accelerating voltage of 80 kV with a 50-μ objective aperture.

Two sets of morphologic experiments were carried out. In the first set, the mucosal surface of hemibladders was exposed to polylysine at concentrations of 2, 8, or 80 μg/ml for 2 hr. In the second set, the mucosal surface of hemibladders was exposed to ^{3}H-polylysine at a concentration of 80 μg/ml for 5, 15, 30, 60, or 120 min. At the end of both experiments, the mucosal surface of the hemibladders was rinsed with 0.25 M sucrose solution for 10 min to remove unbound polybase, and the hemibladders were placed in fixative.

Materials

Poly-L-lysine HBr (mol wt ≅ 140,000) was obtained from Yeda Ltd., Israel, Pilot Chemicals, Inc. and Miles Laboratories, Inc. The polymer was converted to the chloride form by passage through a Bio-Gel A-5m column. The polymer was eluted with 1.0 M NaCl – 2.4 mM $NaHCO_3$ solutions. The fractions corresponding to the external volume of the column were collected, pooled, and then dialyzed for 2 to 3 days against frog-Ringer's solution. The following materials were obtained commercially and used without further purification: amphotericin B as the dry powder from E. R. Squibb & Sons, Pitressin® from Parke, Davis & Co., d-aldosterone from California Biochemical Co., Bio-Gel A-5 m from Bio-Rad Laboratories, and ^{22}Na and ^{24}Na from New England Nuclear Corp.

Results

Effects of Polylysine on SCC

After overnight preincubation as described above, polylysine solution or an equal volume of the diluent (frog-Ringer's solution) was added to the mucosal solutions of each member of a pair of hemibladders to final concentrations of 2, 8, or 80 μg/ml. The bladders were maintained at null voltage throughout the experiment except for the brief period needed to record the open-circuit transepithelial PD. Two hours after the addition of polylysine or diluent, all hemibladders were challenged with vasopressin (final concentration of 100 mU/ml in the serosal solutions), and the SCC and PD were recorded for an additional hour. At a concentration of 2 μg/ml, the polybase had no effect on SCC or PD over a 2-hr period and had no effect on the response to vasopressin (Fig. 1). At a concentration of 8 μg/ml, the PD declined almost immediately and stabilized at a level of 66 ± 8% of the control value (Fig. 2). The polymer depressed the SCC to 82 ± 5% of the control value. The response to vasopressin, however, was not significantly reduced. At a concentration of 80 μg/ml, there was a curvilinear fall in both SCC and PD without a detectable latent period (Fig. 3). As the fall in PD (21 ± 3% of the baseline value) exceeded the fall

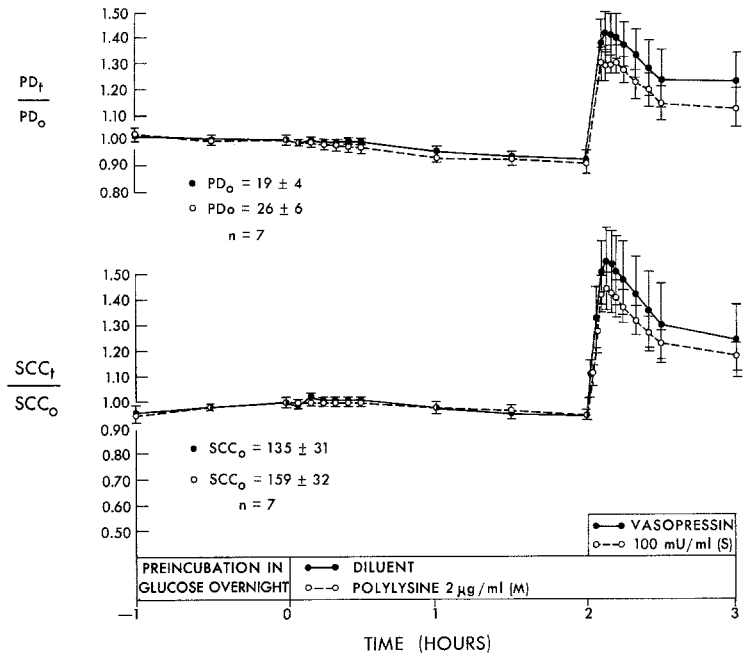
PDt/PDo
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
• PDo = 19 ± 4
o PDo = 26 ± 6
n = 7
SCCt/SCCo
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
• SCCo = 135 ± 31
o SCCo = 159 ± 32
n = 7
VASOPRESSIN
o—o 100 mU/ml (S)
PREINCUBATION IN GLUCOSE OVERNIGHT
DILUENT
o—o POLYLYSINE 2 μg/ml (M)
−1
0
1
2
3
TIME (HOURS)

Fig. 1

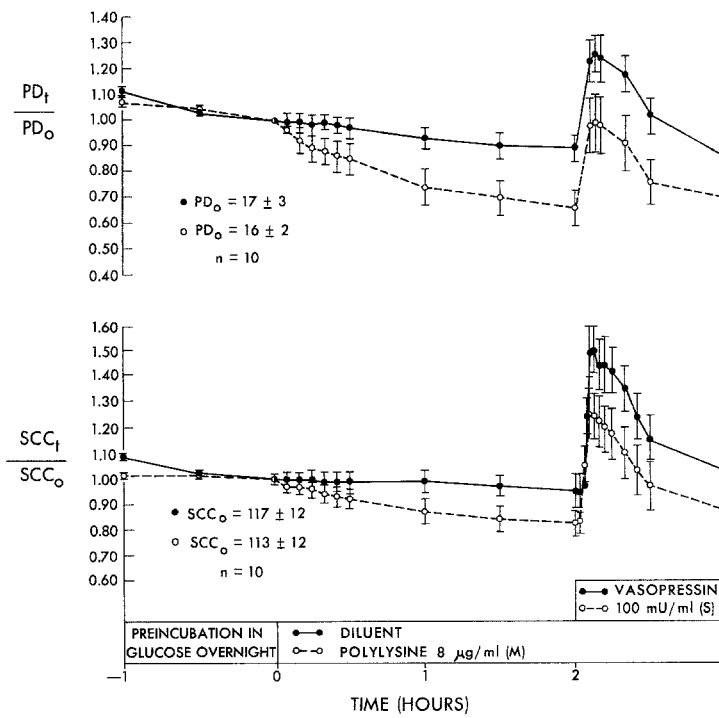
PDt/PDo
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
0.50
0.40
• PDo = 17 ± 3
o PDo = 16 ± 2
n = 10
SCCt/SCCo
1.60
1.50
1.40
1.30
1.20
1.10
1.00
0.90
0.80
0.70
0.60
• SCCo = 117 ± 12
o SCCo = 113 ± 12
n = 10
VASOPRESSIN
o—o 100 mU/ml (S)
PREINCUBATION IN GLUCOSE OVERNIGHT
DILUENT
o—o POLYLYSINE 8 μg/ml (M)
−1
0
1
2
3
TIME (HOURS)

Fig. 2

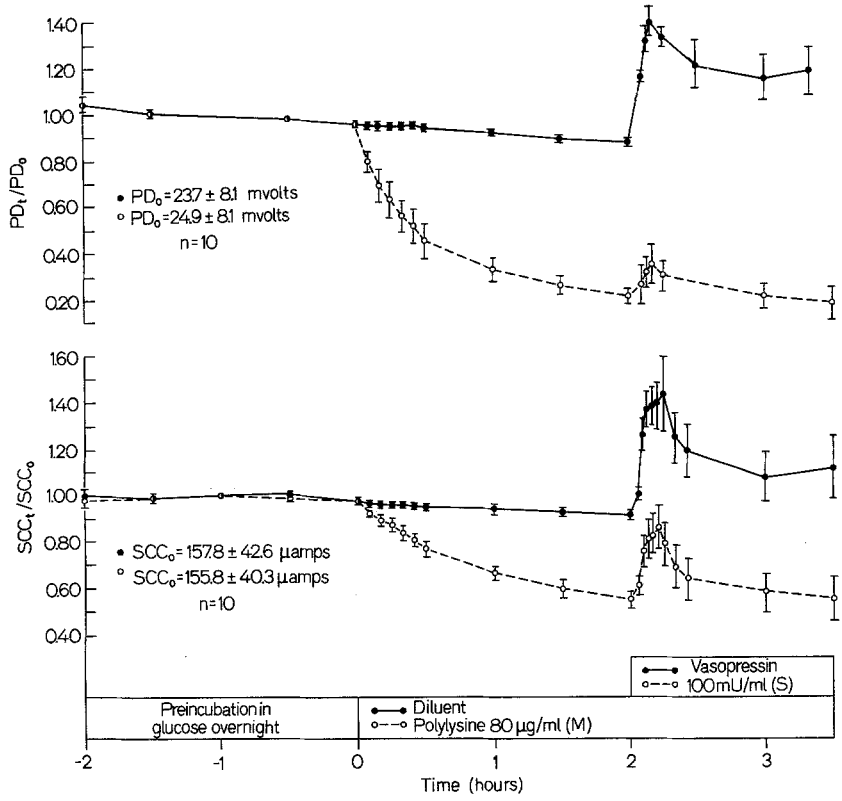

Fig. 3. Effect of polylysine (80 μg/ml) and vasopressin (100 mU/ml) on the SCC and PD of paired toad bladders. See the legend of Fig. 1 for the conventions used in this figure

in SCC (54 ± 4 % of the baseline value), total conductance rose significantly. The absolute response to vasopressin was markedly attenuated, particularly the change in PD. On a relative scale, however, the percentage increase in SCC was the same in the control and polybase-treated hemibladders (i.e., SCC increased by 159 % in both populations). In contrast to these effects, exposure of the *serosal surface* of the toad bladder to polylysine in the medium at a concentration of 80 μg/ml had no effect on either SCC or PD.

Fig. 1. Effect of polylysine (2 μg/ml) and vasopressin (100 mU/ml) on the SCC and PD of paired toad bladders. The additions were made at the times indicated by the boundaries of each block. PD_t/PD_0 denotes the potential difference at time "t" divided by that recorded at time zero in each hemibladder. SCC_t/SCC_0 denotes the ratio of the short-circuit currents recorded at time "t" and time zero. Each point and vertical line represents the mean ± S.E. "n" denotes the number of pairs of hemibladders. PD_0 and SCC_0 denote the absolute PD and SCC at time zero in units of mV and μA/2.54 cm^2 and are given as the mean ± S.E.

Fig. 2. Effect of polylysine (8 μg/ml) and vasopressin (100 mU/ml) on the SCC and PD of paired toad bladders. See the legend of Fig. 1 for the conventions used in this figure

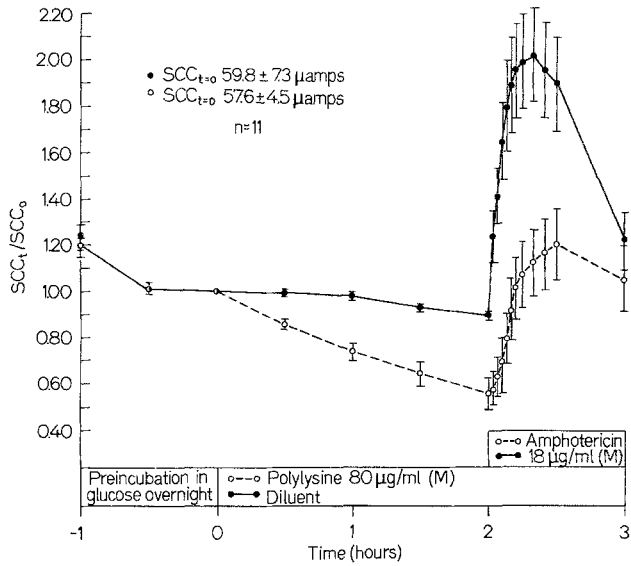

Fig. 4. Effect of polylysine (80 μg/ml) and amphotericin B (18 μg/ml) on the SCC and PD of paired toad bladders. See the legend of Fig. 1 for the conventions used in this figure

Lichtenstein and Leaf [23] proposed that amphotericin B and vasopressin act at the same subcellular site and stimulate active Na^+ transport across the toad bladder by facilitating the inward movement of Na^+ at the apical surface of the epithelial cell layer. Therefore, we measured the SCC response to amphotericin B in polylysine-treated bladders and compared the results to those seen with vasopressin. The design of the experiments was identical to those experiments with vasopressin. Polylysine was added to the mucosal medium of one of each pair of hemibladders to a final concentration of 80 μg/ml; 2 hr later, amphotericin B (final concentration, 18 μg/ml) was added to the mucosal media of all hemibladders. As shown in Fig. 4, the polybase caused a 44 ± 6% fall in SCC. The response to amphotericin B was qualitatively similar to that seen with vasopressin. The absolute response was significantly greater in the control preparations, but the relative increments were virtually the same. The rise in SCC was 224% in control hemibladders and 216% in treated hemibladders. These results indicate that the transport capacity of the polylysine-resistant portion of the epithelium was normal.

Mineralocorticoids provide an alternative means of stimulating Na^+ transport in the toad bladder system. Two proposals have been advanced on the penultimate site of steroid hormone action: 1) that aldosterone acts by increasing the Na^+ conductance of the mucosal or apical surface cell membrane [5, 33], and 2) that aldosterone stimulates an increased

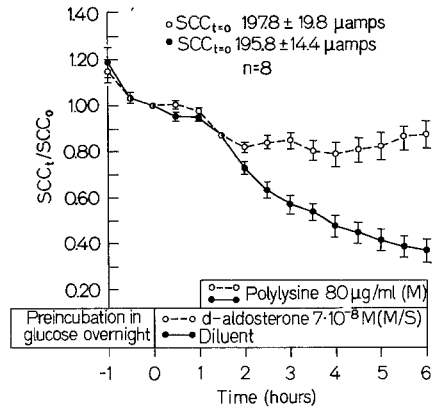

Fig. 5. Effect of polylysine (80 μg/ml) and d-aldosterone (7×10^{-8} M) on the SCC and PD of paired toad bladders. See the legend of Fig. 1 for the conventions used in this figure

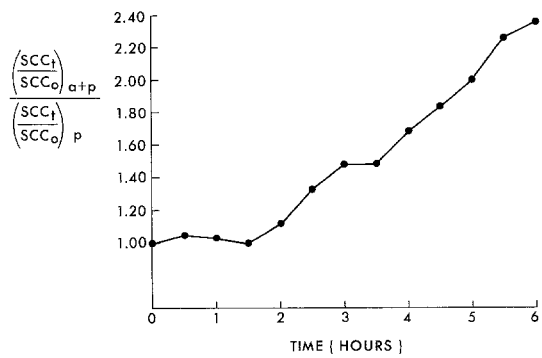

Fig. 6. Response to d-aldosterone (7×10^{-8} M) in the presence of polylysine (80 μg/ml). $(SCC_t/SCC_0)\,a+p$ denotes the ratio of short-circuit currents at time "t" and time zero in the hemibladders exposed to aldosterone and polylysine. $(SCC_t/SCC_0)\,p$ denotes the equivalent short-circuit current ratios in the hemibladders exposed only to polylysine. The mean values were taken from the data in Fig. 5

synthesis of the high energy intermediate – probably ATP – that is the proximate energy donor for the Na^+ pump located in or near the basal-lateral surface membranes [12, 14]. If the polybase acts solely on the apical surface membrane, it should impair the response to aldosterone at that site but not at the Na^+ pump site.

Pairs of hemibladders were preincubated as described above, and one of each pair was exposed to d-aldosterone (final concentration, 7×10^{-8} M in both serosal and mucosal solutions) at time zero. One hour later, polylysine (final concentration, 80 μg/ml) was added to the mucosal solutions of both hemibladders. In steroid-free media, the polybase produced the usual curvilinear decline in SCC (Fig. 5). Aldosterone counteracted this effect almost completely. The difference in SCC at 6 hr

is significant at the 0.1 % level. Moreover, if the response to aldosterone is measured as the paired difference in the SCC ratios, a normal aldosterone-response curve is generated both in amplitude and in the characteristic time-course (Fig. 6). It appears, therefore, that polylysine does not impair the response to d-aldosterone despite its inhibitory effect on the baseline SCC.

SCC and Na^+ Flux

The profound changes in total conductance produced by polylysine raised the possibility that the SCC would not serve as a quantitative measure of active Na^+ transport under these conditions. Simultaneous isotope flux and SCC measurements were made to check this point.

The results of simultaneous bidirectional ^{22}Na and ^{24}Na flux studies are shown in Fig. 7. The radioisotopes were added to the media 2 hr before the addition of the polybase to the mucosal media. The flux and SCC measurements were made over a 3-hr period. The net flux of Na^+ and SCC did not differ significantly in any of the periods studied either before or after the addition of polylysine ($p > 0.05$ in all periods). Thus, the decline in SCC produced by the polybase is ascribable to a corresponding decline in active Na^+ transport. The decline in active Na^+ transport is accompanied by a striking increase in passive permeability to Na^+. This effect is indicated by the greater than twofold increase in unidirectional

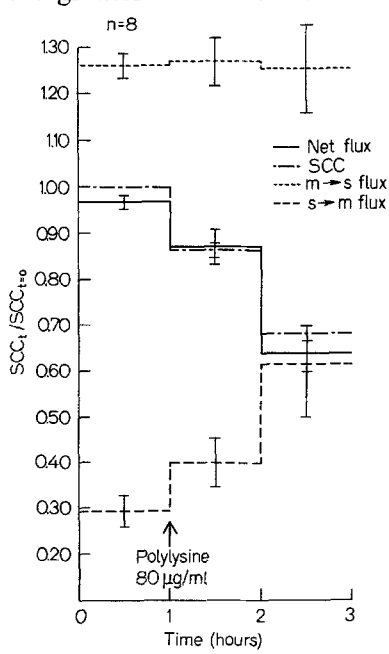

Fig. 7. Effect of polylysine (80 µg/ml) on Na^+ flux and SCC. All fluxes are expressed as a fraction of the simultaneous SCC_0 value. The vertical lines represent ± S.E.

flux of Na^+ from serosa to mucosa. The unidirectional flux from mucosa to serosa remained relatively constant throughout the period of study, indicating that the increase in passive Na^+ conductance was quantitatively offset by an equivalent decrease in active transport.

Effects of Polylysine on Water Flow

The characteristic impermeability of the toad bladder to water in the presence of large osmotic gradients (mucosal side dilute) has been attributed to a porous barrier in or near the apical surface of the epithelial cells [22]. The presumed site of action of vasopressin or its intermediates is also on this hypothetical barrier. We therefore tested the effect of polylysine, with and without vasopressin in the system, on osmotic water flow.

Paired hemibladders were mounted in flow chambers, and water flow was measured under the influence of a fixed osmotic gradient (serosal side, 235 mosm/liter; mucosal side, 23.5 mosm/liter). Polylysine (80 μg/ml) was added to the mucosal solution of one of each pair after a 30-min control period. Two hours after the addition of the polybase, vasopressin (100 mU/ml) was added to the serosal media of all hemibladders. Thirty minutes later, all of the solutions (serosal and mucosal) were exchanged for fresh Ringer's solution at the same osmotic gradient. One hour after this exchange, all of the hemibladders were rechallenged with vasopressin (100 mU/ml). A typical response to this sequence of additions is shown in Fig. 8, and the averaged response in 10 pairs of hemibladders is shown in Fig. 9. Before the addition of vasopressin, mean water flow was 0.29 μliter/min in the controls and 1.47 μliters/min in the polybase-treated

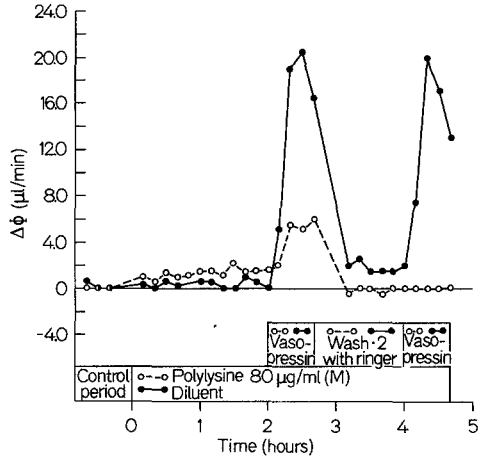

Fig. 8. Effect of polylysine (80 μg/ml) and vasopressin (100 mU/ml) on osmotic flow of water in a pair of toad bladders. The rate of water flow ($\Delta\phi$) is expressed in units of μliters/min per 7 cm^2

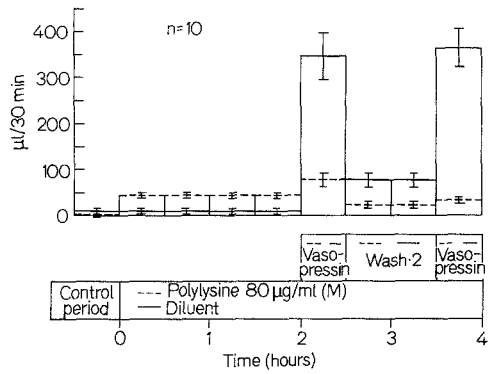

Fig. 9. Effect of polylysine (80 µg/ml) and vasopressin (100 mU/ml) on osmotic flow of water in paired toad bladders. The rate of water flow is expressed in units of µliters/30 min per 7 cm^2. The height of each block indicates the mean rate of water flow and the vertical lines represent ± S.E.

hemibladders. The cumulative 2-hr water flows were 35 ± 11 µliters in the controls and 177 ± 16 µliters in the treated hemibladders ($p < 0.001$). Polylysine produced a small but definite increase in permeability of the bladder epithelium to water. The response to vasopressin, however, was strongly inhibited by the polybase. In the control bladders, water flow was 11.5 ± 1.7 µliters/min during the first 30-min exposure to vasopressin, and 12.1 ± 1.3 µliters/min during the second 30-min exposure. In the polybase-treated bladders, the corresponding results were 2.5 ± 0.5 µliters/min and 1.1 ± 0.4 µliters/min. Exposure of the apical surface to the polybase, therefore, virtually eliminated the water flow response to vasopressin, and responsiveness was not restored by removal of the polybase from the mucosal media for 1.5 hr.

Radioautographic Localization of ^{3}H-Polylysine

At pH ≅ 7.0 and a mol wt of 100,000, polylysine has a net positive charge of about 650. Because of its large size and net charge, this polymer

Fig. 10 A–C. Radioautographs of bladders exposed on their mucosal surfaces to 80 µg ^{3}H-polylysine for 2 hr. Fixation: 1% osmium tetroxide [28]; Embedment: Araldite, 1-µ sections; Photographic emulsion: Kodak NTB 2; Stain: methylene blue-methylene azure A [1]. × 540. A) Section of a contracted bladder. The epithelial cell layer is highly convoluted. Photographic grains outline apical surfaces of mucosal epithelial cells which were in direct contact with ^{3}H-polylysine. In addition, radioautographic grains are seen overlying densely stained clumped material in some cells. B) Section of a partially stretched bladder. Only the apical surface of superficial epithelial cells and occasional clumps within these cells are labelled. C) Section of a stretched bladder. Note that many superficial epithelial cells are lysed. Basal cells are not labelled

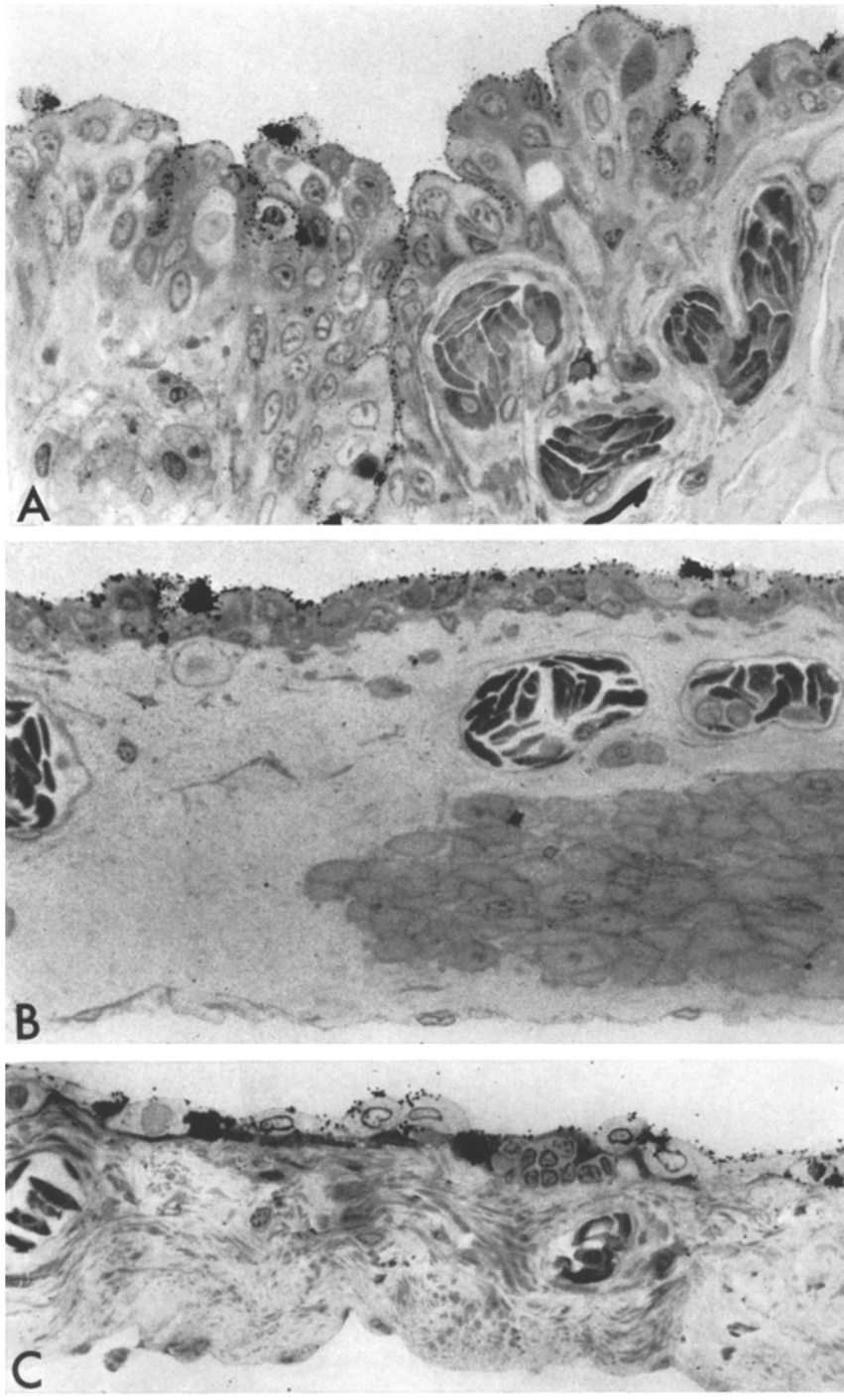

Fig. 10 A – C

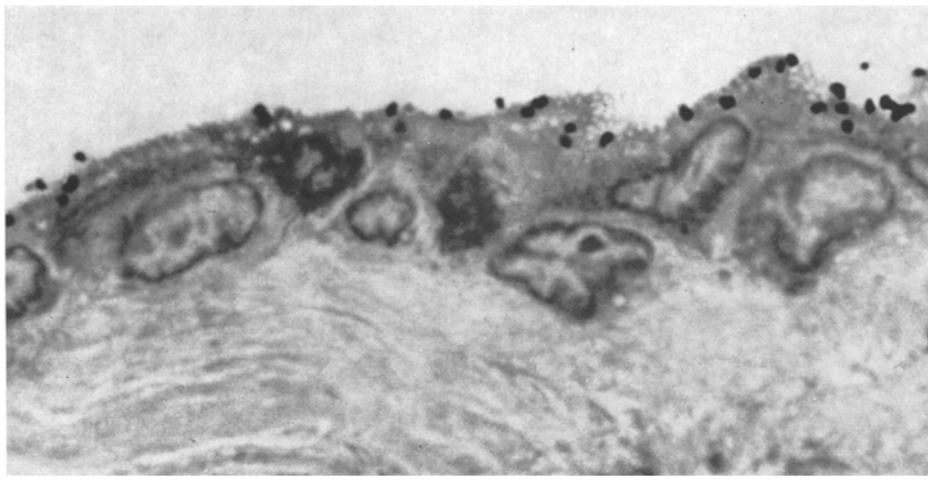

Fig. 11. High-power view of a radioautograph of a section similar to Fig. 10C. Grains overlie the region of the apical plasma membrane. × 2,600

should bind to the apical surface of the epithelial cells and be excluded from their interior. This inference was tested by radioautography.

Radioautographs obtained after exposure of stretched and unstretched hemibladders for 2 hr to ^{3}H-polylysine (80 μg/ml) from the mucosal side are shown in Figs. 10 and 11. In the contracted hemibladder (Fig. 10A), ^{3}H-polylysine was bound along the apical surface and outlined the extensive convolutions of the lumenal surface of the epithelium. No grains were seen in the interior of the epithelial cells, indicating that the polymer did not penetrate the apical plasma membrane. In the stretched bladder (Fig. 10A and C), the label was also distributed along the apical surface of the superficial epithelial cells and in clumps of densely stained material at the apical borders of a few of these cells. In one instance where the continuity of the surface layer was broken, grains were found in the subepithelial connective tissue only in the area immediately beneath the break. Selective localization to the apical membrane is demonstrated in

Fig. 12A–C. Radioautographs of bladders exposed on their mucosal surface to 80 μg/ml of ^{3}H-polylysine for varied lengths of time. Fixation: 1% osmium tetroxide; Embedment: Araldite; Photographic emulsion: Kodak NTB 2; Stain: methylene blue-methylene azure A. × 650. A) Section of a bladder exposed for 30 min to ^{3}H-polylysine and subsequently exposed to Kodak NTB 2 emulsion for $7\frac{1}{2}$ months. Only a few scattered grains are visible. B) Section of a bladder exposed for 1 hr to ^{3}H-polylysine and subsequently exposed to Kodak NTB 2 emulsion for $7\frac{1}{2}$ months. Photographic grains outline the apical surface of superficial epithelial cells. C) Section of a bladder exposed for 2 hr to ^{3}H-polylysine and subsequently exposed to Kodak NTB 2 emulsion for 1 month. In comparison with Fig. 12B, note the greater number of enlarged pale cells and the clumps of dense material within some cells

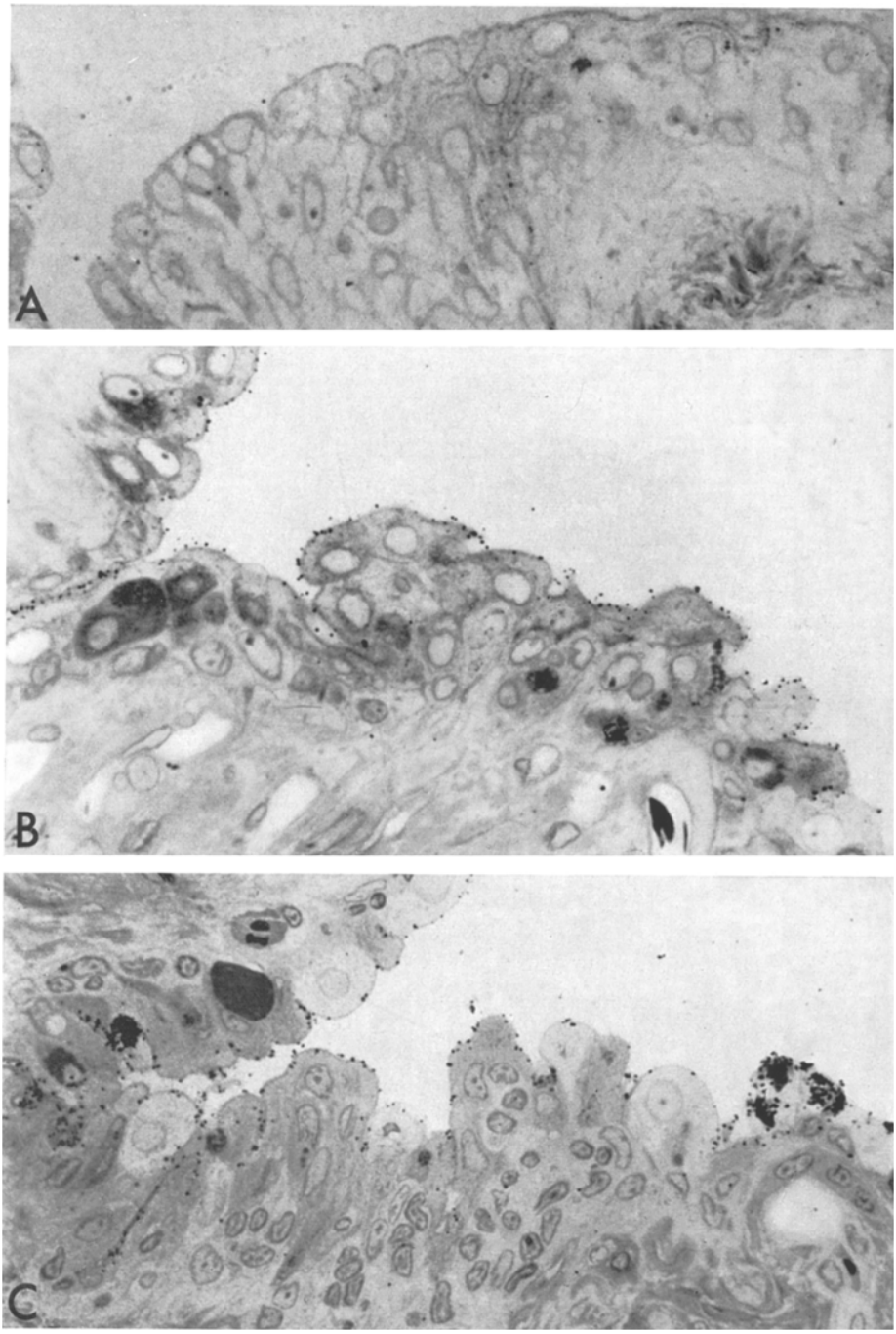

Fig. 12A–C

the high power view of a radioautograph of a stretched hemibladder in Fig. 11. The grains are clearly limited to the apical boundary. At this level of resolution, it is impossible to distinguish between binding to the plasma membrane or to the overlying mucous coat.

Bladder sections taken after exposure to ^{3}H-polylysine for 5, 15, or 30 min did not show significant labelling, suggesting that the binding process was relatively slow. As shown in Fig. 12A, no grains were seen in hemibladders exposed for 30 min. Significant numbers of grains limited to the apical margin of the epithelium were seen, however, after exposure to the tritiated polybase for 1 hr (Fig. 12B). The quantity bound was notably less than after 2 hr of exposure to the labelled material, although the pattern of distribution of the grains was identical (Fig. 12C). The grain density is about the same in Figs. 12B and C, but the duration of exposure to the emulsion was 1 month in Fig. 12C and $7\frac{1}{2}$ months in Fig. 12B. The difference in the time of exposure to the emulsion required to give equivalent grain densities indicates that binding of the polybase increased significantly during the 2nd hr of contact with ^{3}H-polylysine. These results are compatible with two possible interpretations: 1) the rate of binding was relatively slow, or 2) more negatively charged sites became exposed during the reaction because of extraction or removal of masking substances (e.g., precipitation of surface mucus by the polybase). That significant interactions between the polymer and the cells in the first minutes of exposure did occur, however, is indicated by the rapid fall in SCC and PD (Fig. 3) and the appearance of morphological changes in epithelial cells as soon as 5 min after exposure to the polybase (*see* below).

Morphological Effects of Polylysine

In Vitro Incubation. The epithelium of the toad bladder consists of four types of cells. The granular or ordinary epithelial cells account for about 75% of the total cell population [19]. Goblet, mitochondria-rich, and basal cells comprise the remainder. In the stretched state, the epithelium

Fig. 13. This bladder was mounted in a chamber in a semicontracted state and was preincubated overnight in frog-Ringer's solution. Its superficial epithelial cells are tall cuboidal in shape. The basal surface of the epithelium has numerous small folds which protrude into the underlying connective tissue. Arrows indicate epithelial nuclei in which the heterochromatin lining the inner surface of the nuclear membrane stains with normal intensity but the nuclear matrix is pale. ×1,450

Fig. 14. Epithelium from a bladder that was preincubated overnight in frog-Ringer's solution. The majority of the epithelial cells stain with normal intensity with toluidine blue. A few "pale" cells (arrows) are less deeply stained but otherwise appear normal. ×1,200

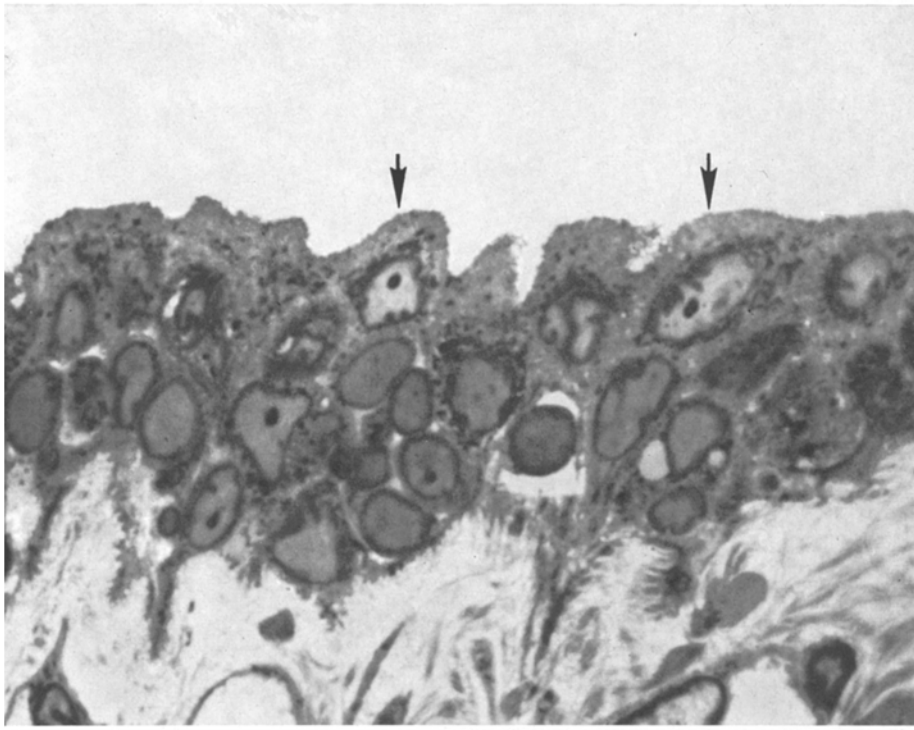

Fig. 13

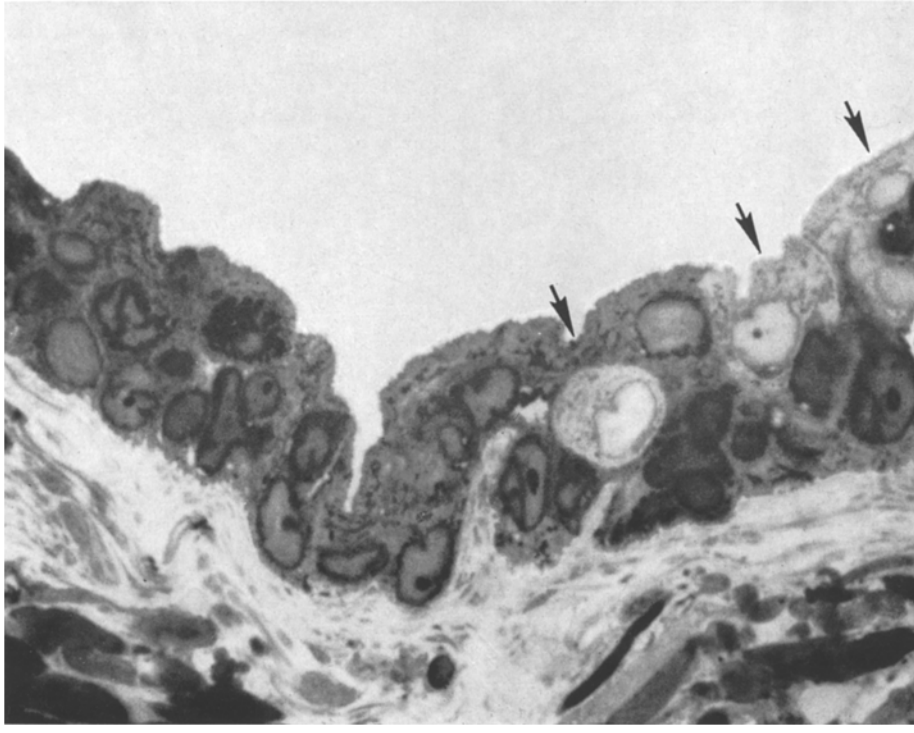

Fig. 14

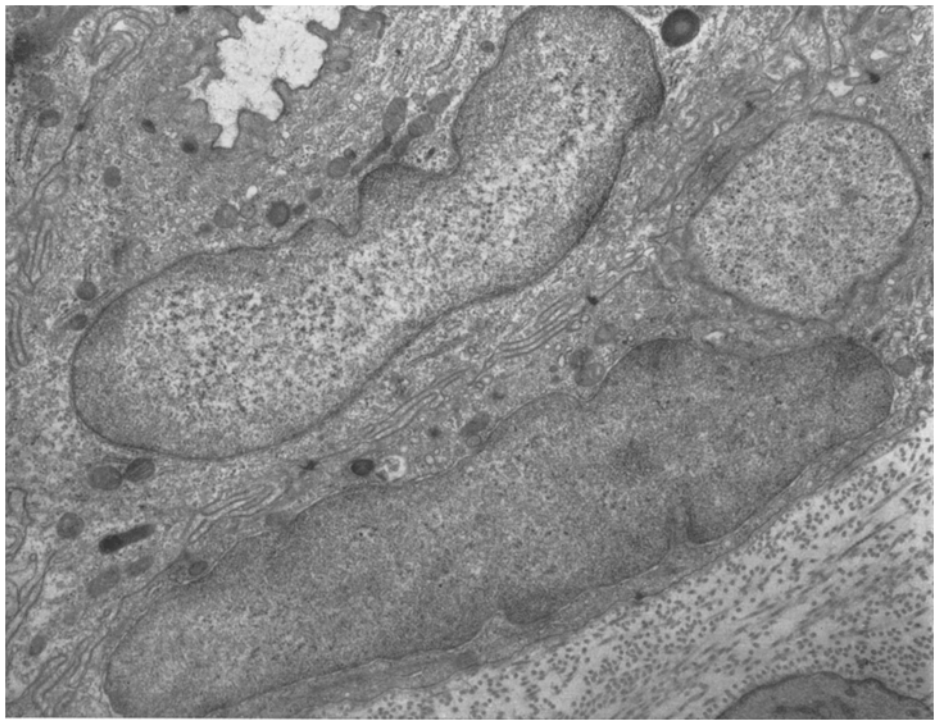

Fig. 15. Epithelial cells in a bladder that was preincubated overnight in frog-Ringer's solution. Two elongated nuclei are shown in the figure. The nucleus of the basal cell has the appearance typical of nuclei fixed with osmium tetroxide; i.e., its chromatin is homogeneously distributed throughout its interior. The matrix of the nucleus of the granular cell above it appears "rarefied"; i.e., clear spaces separate fibrillar and granular components of its matrix. In other respects it appears normal. × 6,600

is one to two cell layers in thickness. Only granular, goblet, and mitochondria-rich cells occur in the superficial layer, and the base of each of these cells has been observed to contact the basement membrane at some point [7]. The basal cells are intercalated between the bases of the cells of the superficial layer. They rest on the epithelial basement membrane and do not form a continuous layer. In the contracted state, the epithelial cells are more columnar in shape. The lumenal and basal surfaces of the epithelium are highly infolded. The folds of the lumenal surface are often difficult to discern by light microscopy because apical surfaces of opposed epithelial cells are pressed closely together (Figs. 10A and 12).

Fig. 16. Two granular epithelial cells are shown in this figure. The cell on the right appears normal. The cell on the left is a "pale" cell. Its mitochondria are less electron-dense than those of the normal cell. In addition, its cytoplasm is relatively deficient in small vesicular components and relatively rich in a fine fibrillar material. × 9,600

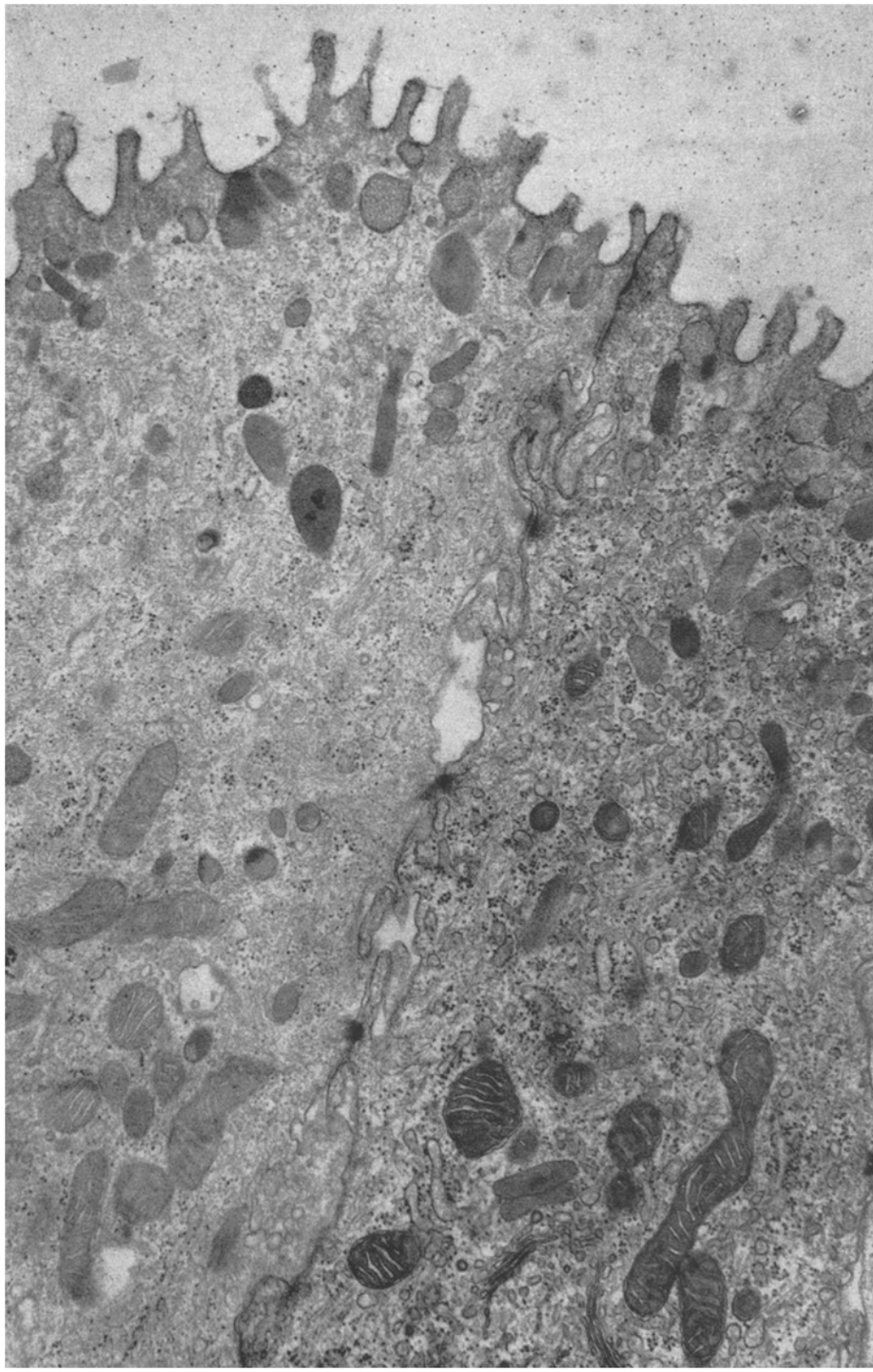

Fig. 16

Detailed descriptions of the ultrastructure of the epithelium have been reported previously [4, 29]. We found that the toad bladder was well preserved in osmium tetroxide-containing fixatives. Therefore, little arti-

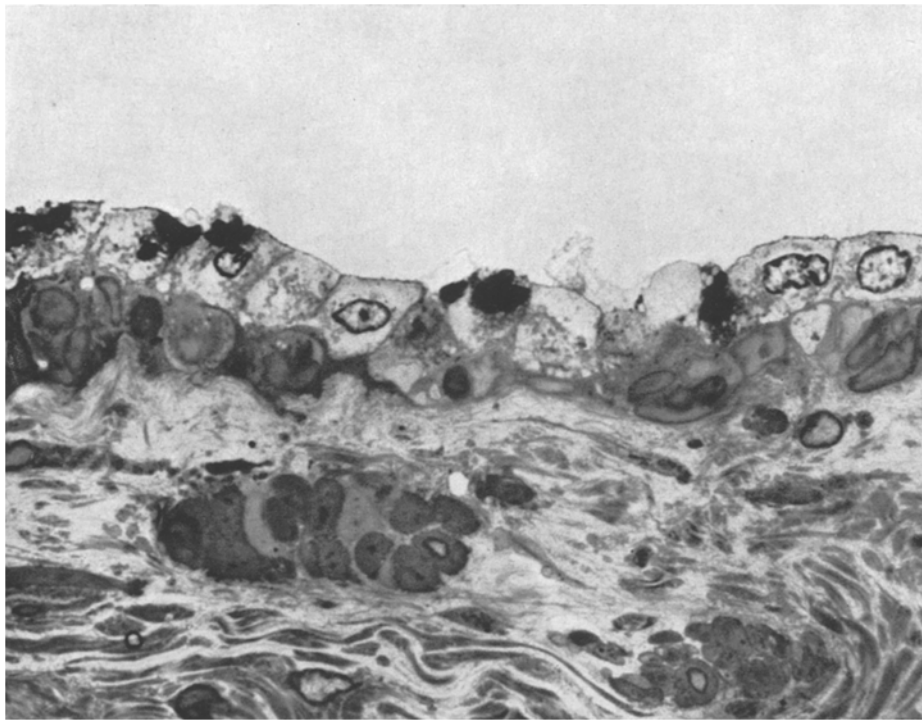

Fig. 17

Figs. 17–21 are light or electron micrographs of bladders that were preincubated overnight in frog-Ringer's solution and then their lumenal surface was exposed to 80 μg/ml of polylysine for 2 hr

Fig. 17. The epithelium of the bladder is two cell-layers thick. The cells in its superficial layer are lysed. Their cytoplasmic matrix is extremely pale and appears to have been extracted. Against the background of the pale matrix are seen filamentous and granular bodies which presumably are remnants of cytoplasmic organelles. Larger deeply stained masses appear in the apical region of some cells. The masses may extend from the apical plasma membrane to the nucleus. The borders of lysed cells are readily discernible, and their plasma membranes appear intact. The nuclear matrix is weakly stained, but remnants of chromatin lining the nuclear membrane stain deeply. The cells in the basal layer appear relatively normal. ×960

Fig. 18. The cell membranes of the superficial lysed cells and the junctions linking them to one another and to the underlying epithelial cells are still intact. Remnants of the mucous coat normally present along the lumenal surface are condensed into a few small clumps. Electron-dense chromatin lines the nuclear membrane of an elongated pycnotic nucleus. Damaged mitochondria are present in the cytoplasm of the lysed cells. Other cytoplasmic organelles are either leached out or too distorted to be identified. The cells of the basal layer appear normal. ×2,700

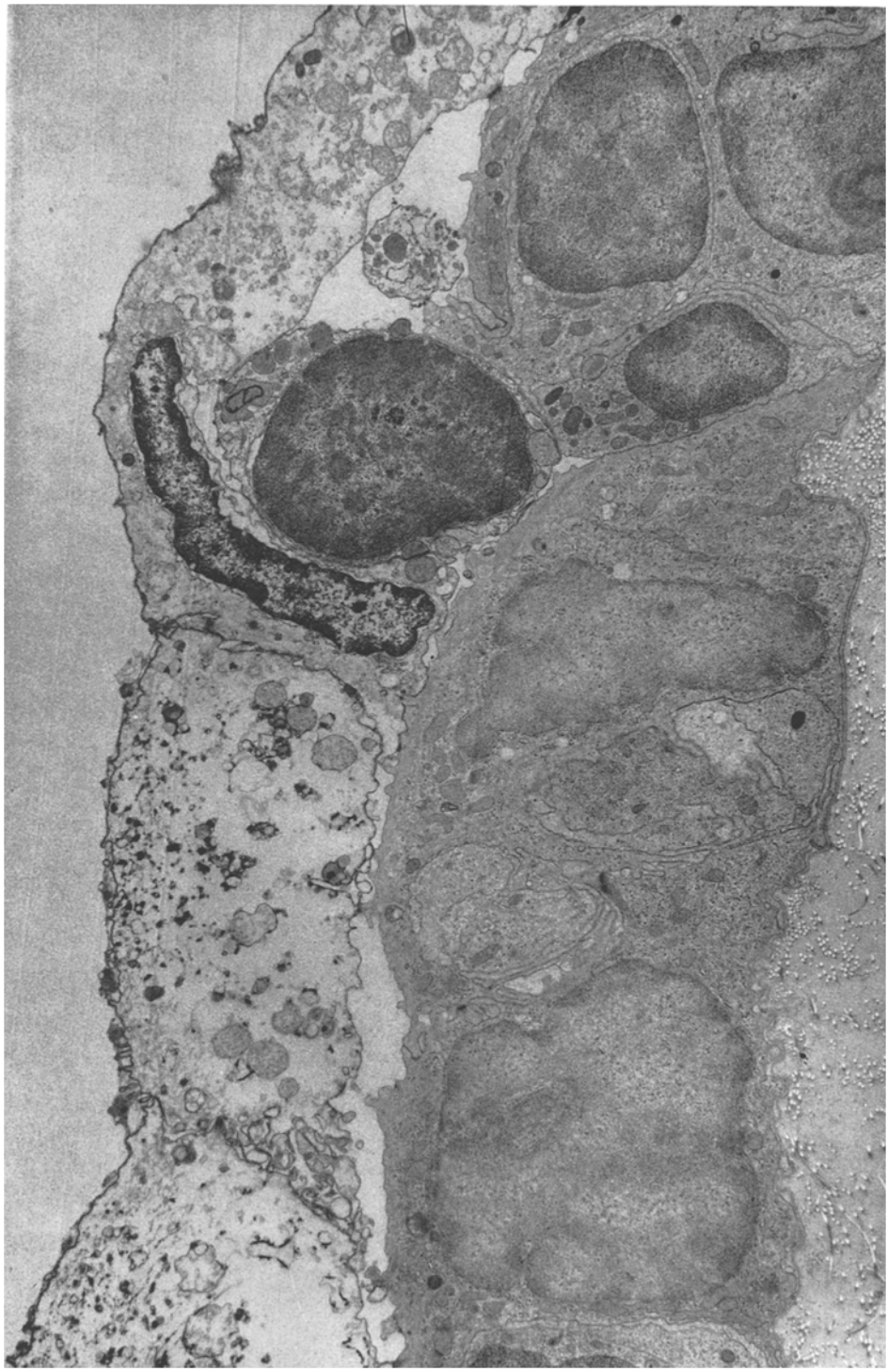

Fig. 18

fact was introduced in the preparation of bladder specimens for electron microscopy.

In one set of experiments, the hemibladders were preincubated for 14 to 16 hr before exposure to the polybase. The effect of preincubation on the morphology of the epithelium, therefore, was studied. In 0.45-μ sections stained with toluidine blue, the nuclear matrix of some cells in the superficial layer appeared paler than normal (Fig. 13), and a few of the superficial cells had an overall pale appearance (Fig. 14). Under electron-microscopic examination, the reduction in affinity of the nuclear matrix for toluidine blue correlated with a "rarefied" appearance of the matrix due to the presence within it of empty spaces (Fig. 15). Cytoplasmic organelles of pale cells lacked their normal electron density (Fig. 16). This was readily observable in the case of the limiting membranes and matrix of mitochondria. In addition, membranous cytoplasmic organelles were reduced in number, and a finely fibrillar substance in which free ribosomes were dispersed appeared in the cytoplasm. Rarefaction of the nuclear matrix was also seen but less frequently in bladders fixed immediately after removal from the toads. Overall, preincubation caused few changes in morphology.

Exposure to Polylysine. Hemibladders were exposed to several concentrations of polylysine (2, 8, or 80 μg/ml) in the mucosal solutions for 2 hr after overnight incubation in frog-Ringer's solution. Exposure to 2 or 8 μg/ml of polylysine had little effect on the morphology of the epithelium. As in the controls after overnight incubation, "pale" cells were found interspersed among normal superficial epithelial cells. In rare instances, single necrotic cells were seen desquamating from the epithelial surface. In contrast, exposure to 80 μg/ml of polylysine produced profound morphologic changes. In stretched bladders, virtually all of the superficial epithelial cells showed extensive damage when examined by light microscopy (Fig. 17). Their plasma membranes were sharply outlined and were for the most part intact. The cytoplasmic matrix was extremely pale and

Fig. 19. The plasma membrane of the lysed superficial epithelial cell shown here is intact but somewhat thickened, and the cell is still attached to its neighbors. Although most of its cytoplasmic components have been leached out, scattered vesicles of rough-surfaced endoplasmic reticulum and distorted mitochondria can still be recognized. The latter are swollen, and their matrix lacks normal electron density. There are empty areas in the center of the nucleus, and abnormally electron-dense chromatin is concentrated along the nuclear membrane. At several points, the outer lamella of the membrane is separated from the nucleus. The mucous coat on the apical surface of the cell is aggregated in clumps. The appearance of the lysed superficial cell contrasts with that of the normal basal cell in the lower left corner of the figure. × 10,000

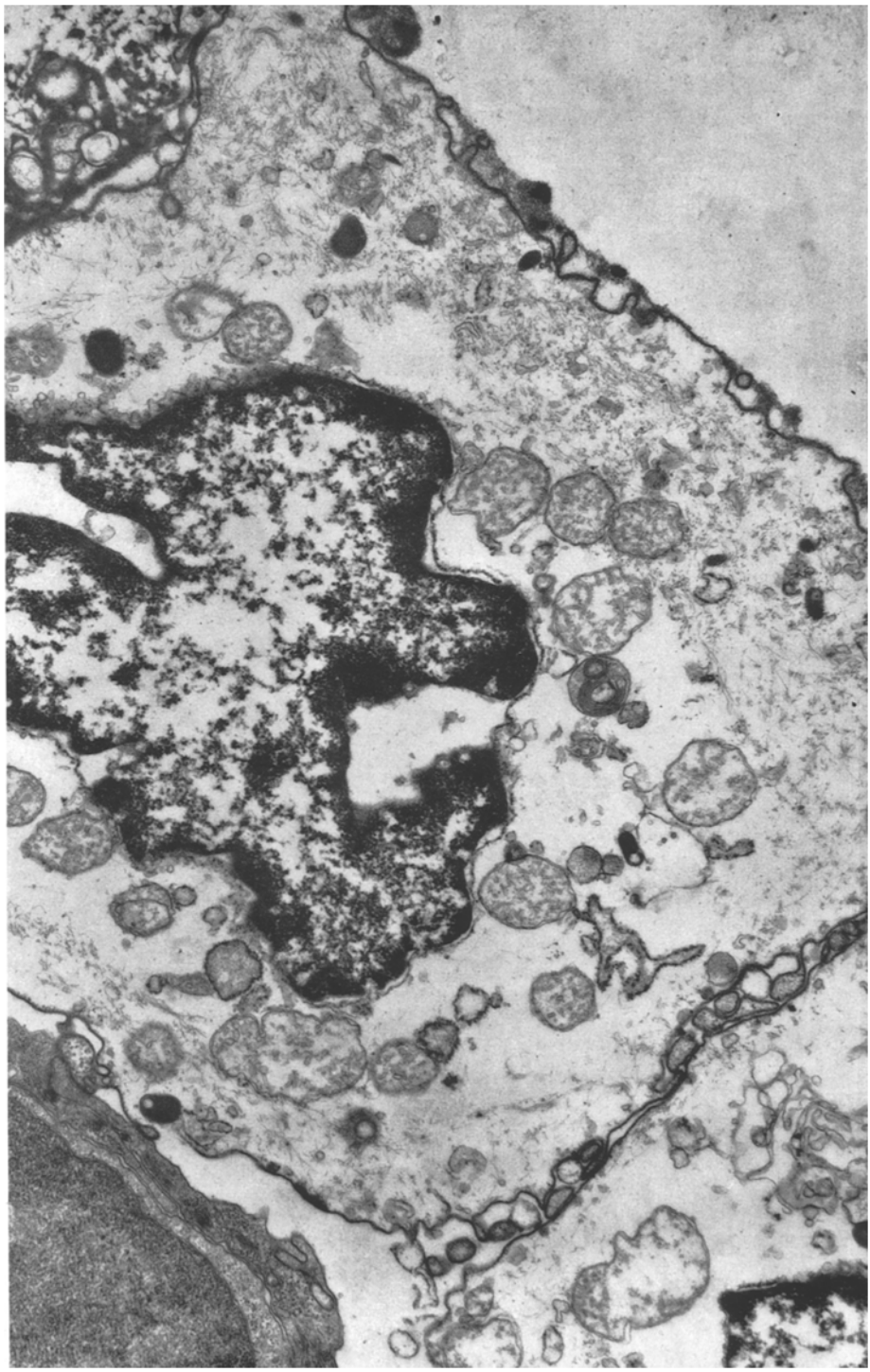

Fig. 19

contained only remnants of the cytoplasmic organelles. In some cells, large densely stained masses appeared near the apical surface and occasionally extended deep into the cytoplasmic matrix. The nuclei were sharply outlined by condensed deeply staining chromatin lining the inner margin of the nuclear membrane.

By electron microscopy, the plasma membrane of the lysed cells, although thickened, was intact and the intercellular junctions were preserved (Figs. 18 and 19). The mucous coat on the lumenal surface was condensed into small clumps. The cytoplasmic matrix was leached in appearance, and contained scattered fine filaments and swollen vesicles of the rough-surfaced endoplasmic reticulum. The mitochondria were swollen and distorted, and showed considerable loss of matrix density. Their limiting membranes were, however, intact. Most of the nuclear content was extracted and dense clumps of chromatin were aggregated along the inner surface of the nuclear membrane. The nuclear membrane persisted, although the intracisternal space was abnormally dilated. The basal cells of the epithelium, in contrast to the lysed cells of the superficial cell layer, remained normal in appearance (Fig. 18).

One of the dense masses seen by light microscopy at the apical margin of lysed cells is shown in an electron micrograph in Fig. 20. It consists of a homogeneous electron-dense material in which an even more dense filamentous network is embedded. The mass also contains membranous inclusions with multiple parallel lamellae. In this section, the dense mass extends from the plasma membrane to the apical pole of the nucleus.

We were especially interested in the possibility that binding of polylysine to the apical surface might produce morphological changes in the apical plasma membrane recognizable by electron microscopy. After exposure for 2 hr to 80 μg/ml of polylysine in the mucosal solution, the cell membrane along all of the surfaces (i.e., apical, lateral, and basal) of the superficial epithelial cells was thickened, apparently because of deposition

Fig. 20. Dense mass in a superficial epithelial cell. The mass consists of a homogeneous matrix material in which are lodged a variety of inclusions. Among these are membranous inclusions, some of which contain multiple parallel lamellae, and a reticulum of electron-opaque filaments. The mass extends from the apical plasma membrane to the apical pole of the nucleus. × 14,400

Fig. 21. Surface regions of adjacent bladder epithelial cells. The cell in the upper part of the figure is in the superficial cell layer; the cell in the lower part is in the basal cell layer. The cytoplasm of the superficial cell is devoid of normal components, and its plasma membrane is thickened. The cytoplasm of the basal cell appears normal and contains vesicles, a lamellated dense body, and numerous ribosomes and fine filaments. Its plasma membrane appears normal. × 35,000

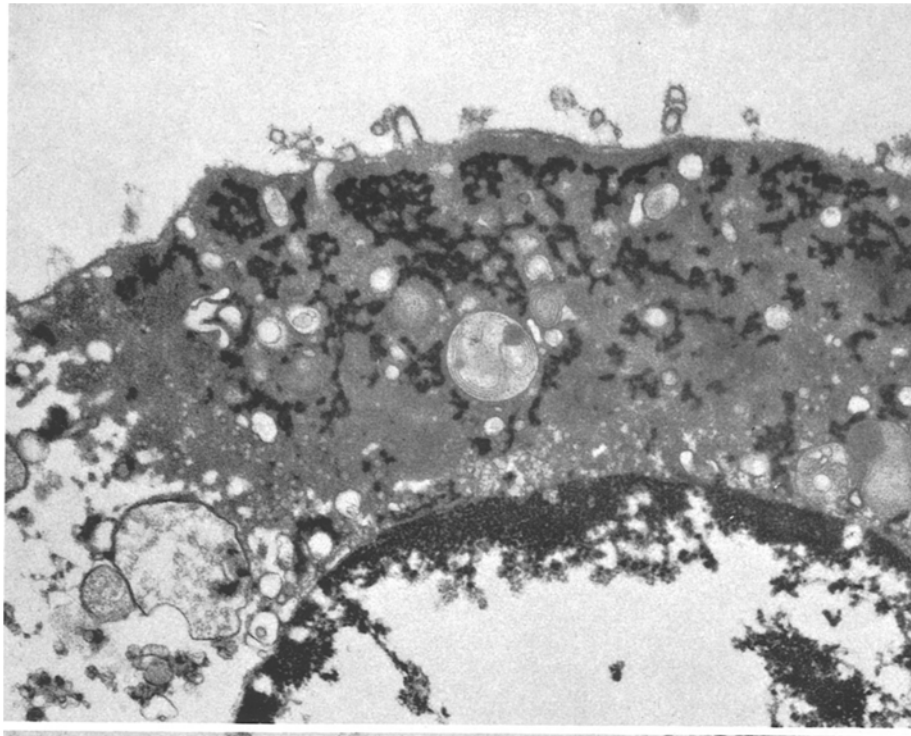

Fig. 20

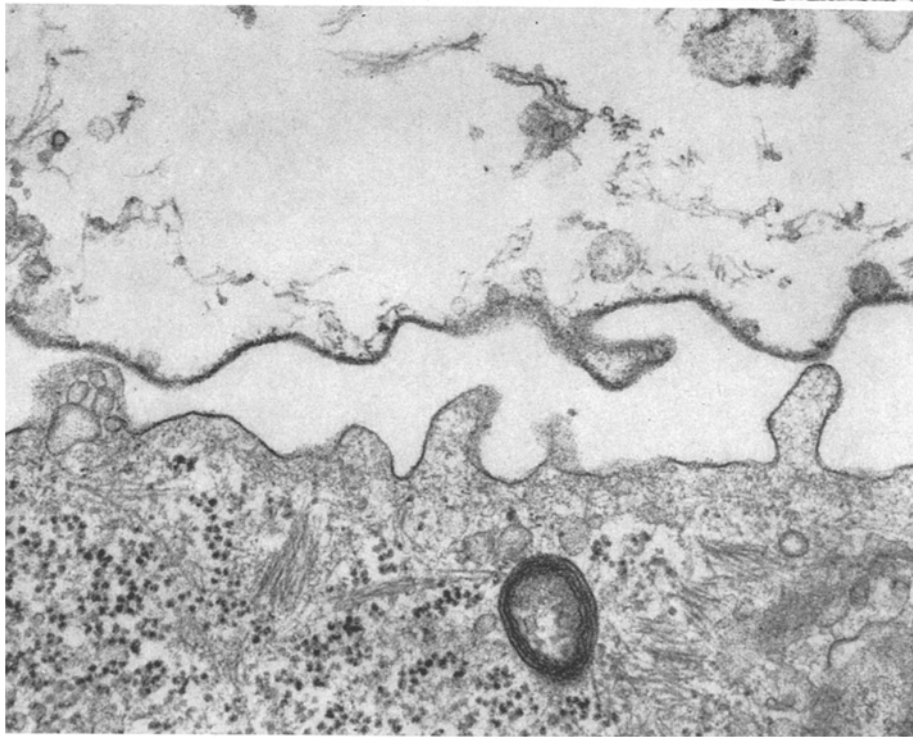

Fig. 21

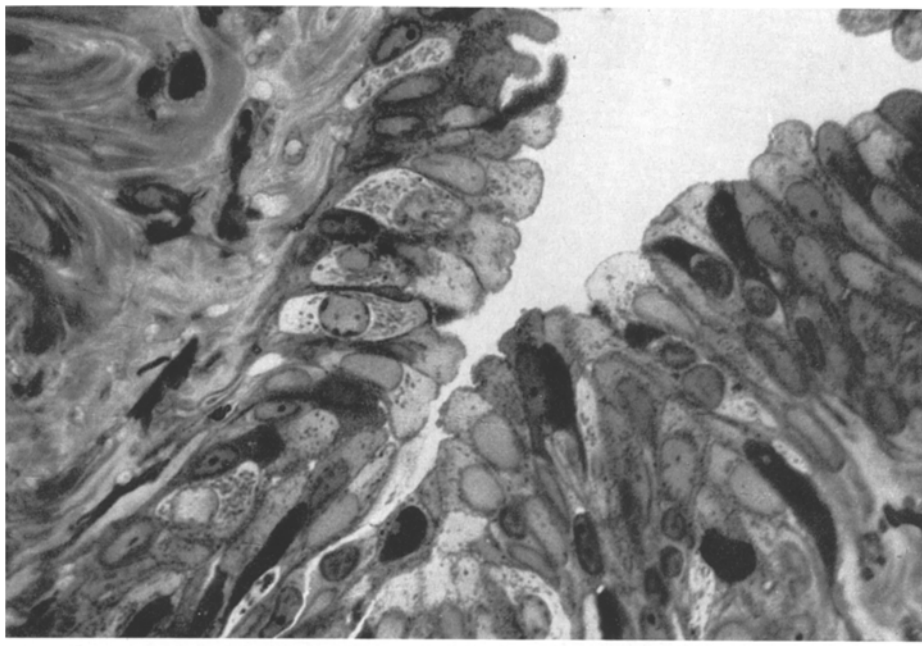

Fig. 22

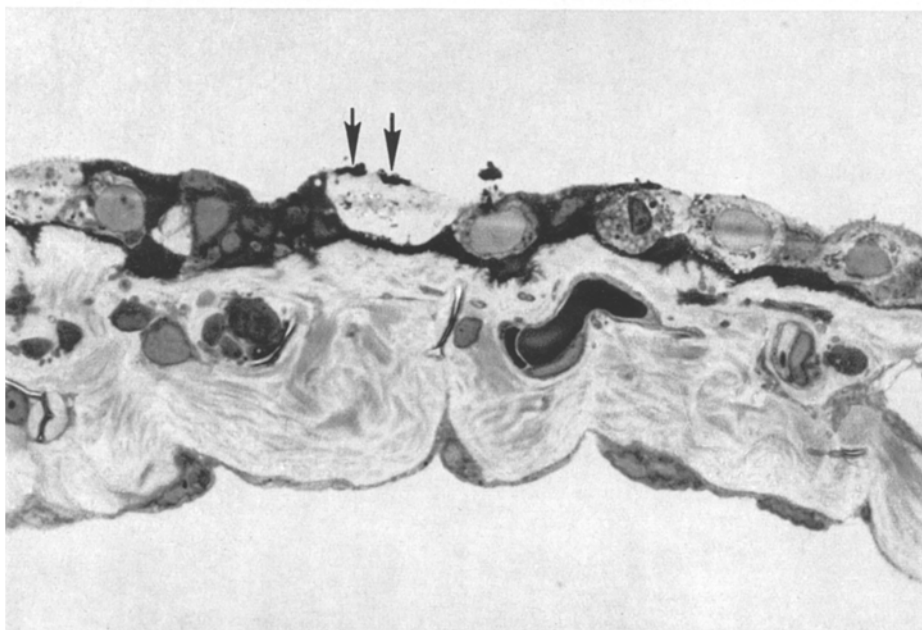

Fig. 23

Fig. 22. Araldite section of a contracted bladder exposed to ^{3}H-polylysine (80 µg/ml) for 2 hr. Scattered among the normal cells are many pale and partially lysed epithelial cells. × 925

Fig. 23. Epithelium of a bladder exposed along its lumenal surface to a solution containing 80 µg/ml of polylysine for 1 hr. There are pale cells and lysed cells in the superficial cell layer among denser cells which appear normal. Two dense masses (arrows) appear at the apical margin of a lysed cell. × 960

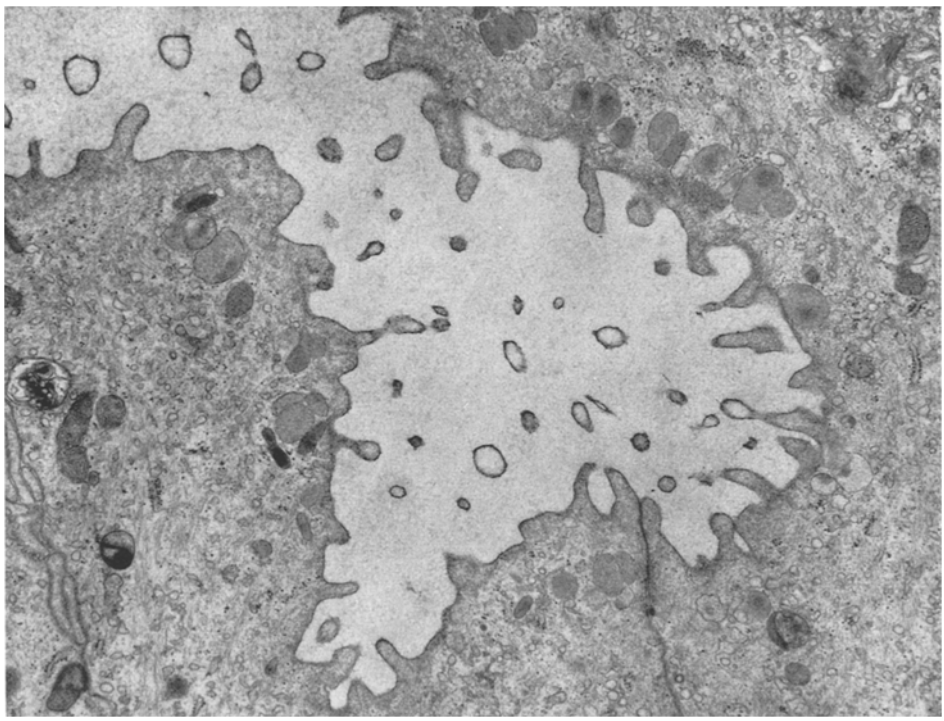

Fig. 24. Lumenal surface of epithelial cells exposed to a solution containing 80 μg/ml of polylysine for 5 min. The apical surface of the granular epithelial cells shown in the figure lacks a mucous coat. The empty vesicles in the lumen near the cells are the swollen tips of microvilli. × 8,400

of a finely fibrillar electron-dense material on its cytoplasmic face (Fig. 21). The unit-membrane structure of the plasma membrane was no longer clearly discernible. Despite the lysis of the cells in the superficial layer, junctions between adjacent cells were preserved, and the cells of this layer were held in their normal position (Fig. 18). The plasma membranes of the underlying basal cells remained normal in appearance (Fig. 21).

The changes described above were seen uniformly in stretched bladders exposed to high concentrations of the polycation. In contracted bladders, the apical surface of the epithelium is deeply infolded as shown in the radioautograph in Fig. 10A. Many superficial cells were apparently protected against the lytic action of polylysine (80 μg/ml in the mucosal solution for 2 hr). Severely damaged cells in the superficial epithelial layer were distributed either singly or in small groups and alternated with cells that appeared relatively normal (Fig. 22). The extent of damage, therefore, was determined in part by the configuration of the epithelial layer during exposure to the polycation.

Time-Course of the Morphological Effects of Polylysine. Hemibladders were exposed to 80 μg/ml of polylysine in the mucosal solution for 5, 15, 30, 60, or 120 min. Under electron-microscopic examination, pale cells were first observed in the epithelium after 5 min of exposure to the polymer. The number of pale cells and the degree of loss of their cytoplasmic density increased in proportion to the duration of exposure. Fully lysed cells were seen after exposure to the polymer for 1 hr, at which time dense masses began to appear at the inner surface of some of the apical margins (Fig. 23). After 2 hr of exposure, many of the cells in the superficial layer had been lysed. The pale cells and lysed cells observed in this set of experiments were identical to those seen in the set exposed to polylysine for 2 hr (*see* above), except that the plasma membranes of lysed cells were not thickened.

Several early effects of exposure to 80 μg/ml polylysine were noted by electron-microscopic examination. In 5 min, the mucous coat on the surface of the epithelium was nearly entirely removed, and microvilli of the superficial cells were swollen and devoid of cytoplasmic matrix (Fig. 24).

Discussion

Our initial assumption that polylysine would bind selectively to the apical surface of the epithelium when added to the mucosal medium was verified by the radioautographic results (Figs. 10–12). The photographic grains marking the location of labelled polycation were aligned along the mucosal border of the epithelium. Some grains were also localized over the dense apical masses, indicating that the masses had incorporated the polycation. These results are in accord with earlier studies on bacteria, erythrocytes, and ascites tumour cells [16, 17, 21]. The anionic binding sites on the apical surface of the cell membranes responsible for binding of polycations have not been identified. Danon, Howe, and Lee [6] implicated sialic acid residues of glycoproteins in the binding of polylysine to the red cell plasma membrane. Other possible contributors to the binding process are the phosphate and sulfate groups of phospholipids and acid polysaccharides [31]. These binding groups may be contributed by the mucous coat, by the lipoprotein core of the plasma membrane, or by both.

In earlier studies on erythrocytes and ascites tumour cells, a variety of morphological changes were found including cellular aggregation, inhibition of mitosis, formation of dense masses at the surface of the cells, and complete lysis [17, 21, 27, 32]. Exposure of the mucosal surface of the toad bladder to high concentrations of polylysine for 2 hr also produced

profound morphological changes such as lysis of epithelial cells (i.e., disappearance of their normal intracellular constituents) and the formation of dense masses at some of their apical margins (Figs. 17–23). The preservation of the normal appearance of the basal cells suggests that the polycation did not penetrate beyond the superficial cell layer. Addition of the polymer to the serosal bathing media had no effect on SCC or PD, perhaps because of a failure of the polybase to penetrate to the level of the basement membrane and beyond.

The number and distribution of the damaged cells in the superficial epithelial layer was a function of the degree of stretch of the bladder during exposure to polylysine. In the stretched state, most or all of the superficial cells were lysed (Fig. 17), whereas in the contracted state many of the superficial cells escaped lysis even after 2 hr of exposure to the polycation at a concentration of 80 μg/ml (Fig. 22). We did not study in detail the events leading to the formation of the dense masses seen in the subapical regions of the superficial epithelial cells after exposure to polylysine. The radioautographs and electron micrographs, however, suggest that the masses consist of clumps of degenerating remnants of cytoplasmic components and interspersed polylysine.

It is probable that the polycation interacted with the mucous coat on the apical membrane since exposure to the polymer resulted in clumping or disappearance of the coat (Figs. 18, 19, and 24). The polycation may also have penetrated into the lipoprotein core of the apical plasma membrane and disrupted its organized molecular array. In either event, the net effect may have been to increase the permeability of the apical plasma membrane to the soluble cytoplasmic constituents. The dramatic changes in the intracellular organelles would then be a consequence of the loss of these constituents. Alternatively, binding of the polymer to the apical surface may have triggered the intracellular release of lysosomal enzymes (e.g., proteases, lecithinases) that attacked the organelles.

Despite the lysis of many cells in the superficial layer, the plasma membranes of the lysed cells were continuous and without signs of rupture (Figs. 18 and 19). In electron micrographs, the intercellular junctions were found to be intact. Since the lysed cells remained in their normal position in the epithelium, we assume that the junctional complexes of their plasma membrane retained some measure of their initial adhesivity.

The available evidence derived from electrophysiological, metabolic, and enzymatic studies supports the model for transcellular Na^+ transport shown in Fig. 25 [2, 15, 20, 34]. In essence, the theory states that the transport system is determined by the unique properties of the cell mem-

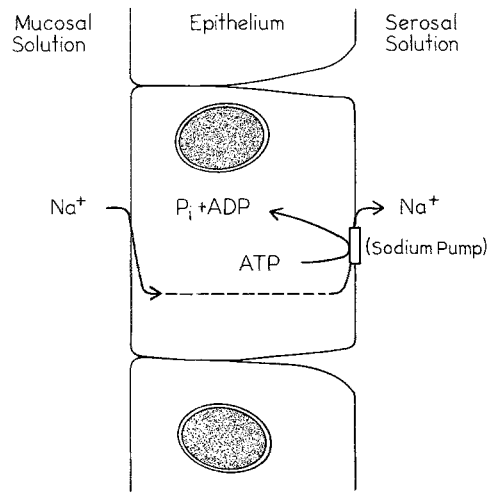

Fig. 25. Hypothetical model of active sodium transport across an epithelial cell layer

brane of the two surfaces, apical and basal-lateral, in series. The apical membrane is selectively permeable to NaCl (and not to K^+), although the kinetics of Na^+ entry may not conform to the predictions of a simple electro-diffusion equation. The apical plasma membrane is also believed to be impermeable to water in the absence of vasopressin or a related neurohypophyseal peptide. In this scheme, Na^+ is extruded against the electrochemical gradient from the interior of the cell into the interepithelial and subepithelial spaces. The Na^+ pump is located in the basal-lateral cell membranes, uses ATP as the proximate energy donor, and has the properties of a Na^+ plus K^+-activated ATPase [34]. It has also been proposed that vasopressin and aldosterone accelerated NaCl transport in this system by facilitating the entry of Na^+ into the cell across the apical boundary [15, 33]. With respect to the mechanism of action of aldosterone, this view is based in part on the assumption that amphotericin B, a polyene with a high affinity for sterols, augments Na^+ transport across the toad bladder by a direct effect on the apical boundary [23]. In studies with ascites tumor cells, Kornguth, Stahmann, and Andersen [21] concluded that polylysine increased the permeability of the cell membrane to solutes. One effect that might have been expected, therefore, would be a biphasic response to the polycation: 1) stimulation of Na^+ transport at low concentrations of polylysine because of an increase in apical permeability, and 2) inhibition of the transport system at high concentrations as a result of cell lysis. At a low concentration of the polycation, no effect on Na^+ transport was seen but inhibition was profound at a high concentration

of the polymer (Figs. 1 and 3). Moreover, the inhibitory effect was monotonic. The close agreement between SCC and net flux of Na^+ ruled out the possibility that failure to detect a stimulatory response to the polymer was a consequence of a discrepancy between current and net flux. The more than twofold increase in serosal to mucosal flux of Na^+ suggests that there was a considerable increase in passive permeability to this ion (Fig. 7). The likeliest explanation for the failure to stimulate Na^+ transport is that the change in apical permeability is nonspecific in character, resulting in losses of intracellular solutes (e.g., ATP, K^+) that are needed for transport activity.

Despite marked inhibition of basal Na^+ transport by polylysine, the time-course and character of the SCC response to aldosterone, vasopressin, and amphotericin B was similar to that in normal paired controls (Figs. 3 – 5). It is possible that binding of polylysine to the apical surface did not impair the response to these agents because they act at the level of metabolic regulation of ATP synthesis or enhancement of the activity of the Na^+ pump. The morphological studies, however, suggest an alternative and probably more plausible explanation. At a concentration of 80 μg/ml in the mucosal medium, polylysine lysed superficial epithelial cells. Both the binding of ^3H-polylysine to the apical surface and the number of lysed cells in the epithelium increased progressively over the 2-hr period of study. The decline in SCC and transepithelial PD correlated approximately with the appearance of the lysed cells. If the breach in apical permeability is a critical phenomenon (i.e., phase transition in the membrane), then the cells that have not been transformed may retain relatively normal responsiveness to the stimulatory agents. In effect, the polycation would reduce the mass of cells involved in the transport process but not the properties of the untransformed cells. In accord with this interpretation is the finding that the peak increase in SCC in response to vasopressin and amphotericin B is proportional to the baseline SCC in the control and polycation-treated hemibladders (Figs. 4 and 5). To characterize the effect of the polymer on the response to aldosterone required a more detailed analysis, as both effects were superimposed over a period of 5 hr. The fractional increase in SCC generated by aldosterone alone was obtained by correcting for the fall in SCC produced by the polymer by dividing the short-circuit current ratio (SCC_t/SCC_0) of the treated group (aldosterone plus polylysine) by that of the control group (polylysine alone) for each 30-min point. The result is shown in Fig. 6. Both the latent period and the rate of rise in SCC is essentially the same as that seen in the normal preparation [30]. Since the results obtained with aldosterone are expressed as

the fractional change in SCC, it is apparent that this result is also consistent with the interpretation of a mixed population of relatively intact and dead cells.

The morphological and Na^+-transport data are consistent with the inference of polymer-induced deletions of cells from the toad bladder epithelium. The results obtained in the studies on osmotic water flow, however, were not entirely in accord with this inference. An all-or-none change in apical permeability should have made these cells grossly leaky to solute and water and should have had no effect on osmotic water flow. Although the effect was small, the polycation produced a statistically significant increase in water flow, suggesting that in some cells there was a differential increase in water compared to solute permeability. In addition, if the intact cells were normal in responsiveness to vasopressin, the increase in osmotic flow of water should have been proportional to the increase in SCC in the polymer-treated and control hemibladders. The results shown in Figs. 3 and 9 indicate clearly that polylysine produced a disproportionate inhibition of the effect of vasopressin on osmotic flow of water. The polycation reduced the effect of vasopressin on SCC to 55% of that in the control hemibladders and on water flow to 9% of that in the controls. Selective inhibition of the antidiuretic action of vasopressin implies either impairment of the action of the intermediate cyclic AMP on the apical membrane or a limitation imposed by a reduction in the effective osmotic gradient, owing to a rise in permeability to solute at the apical surface of the intact cells. The latter explanation is probably correct as the experiments on osmotic flow of water were carried out in volume chambers with large orifice diameters (i.e., 7 cm^2) which necessitated stretching the hemibladders prior to exposure to the polybase. As shown in Fig. 17, all of the surface epithelial cells in the stretched bladder are lysed in 2 hr. Lysis presumably involves a marked increase in the permeability of the apical plasma membrane to solutes.

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References

1. Bélanger, L. F. 1961. Staining processed radioautographs. *Stain Technol.* **36**:313.
2. Bricker, N. S., T. Biber, and H. H. Ussing. 1963. Exposure of the isolated frog skin to high potassium concentrations at the internal surface. I. Bioelectric phenomena and sodium transport. *J. Clin. Invest.* **42**:88.
3. Caulfield, J. B. 1957. Effect of varying the vehicle for OsO_4 in tissue fixation. *J. Biophys. Biochem. Cytol.* **3**:827.

4. Choi, J. K. 1963. The fine structure of the urinary bladder of the toad, *Bufo marinus*. *J. Cell Biol.* **16**:53.
5. Crabbé, J. 1967. Suppression by amphotericin B of the effect exerted by aldosterone on active sodium transport. *Arch. Intern. Physiol. Biochim.* **75**:342.
6. Danon, D., C. Howe, and L. T. Lee. 1965. Interaction of polylysine with soluble components of human erythrocyte membranes. *Biochim. Biophys. Acta* **101**:201.
7. Di Bona, D. R., M. M. Civan, and A. Leaf. 1969. The anatomic site of the transepithelial permeability barriers of toad bladder. *J. Cell Biol.* **40**:1.
8. Dubin, T. D. 1960. The assay and characterization of amines by means of 2,4-dinitrofluorobenzene. *J. Biol. Chem.* **235**:783.
9. Edelman, I. S., R. Bogoroch, and G. A. Porter. 1963. On the mechanism of action of aldosterone: The role of protein synthesis. *Proc. Nat. Acad. Sci., Wash.* **50**:1169.
10. – – – 1964. Specific action of aldosterone on RNA synthesis. *Trans. Ass. Am. Physins.* **77**:307.
11. – M. J. Petersen, and P. F. Gulyassy. 1964. Kinetic analysis of the antidiuretic action of vasopressin and adenosine 3′,5′-monophosphate. *J. Clin. Invest.* **43**:2185.
12. Fanestil, D. D., G. A. Porter, and I. S. Edelman. 1967. Aldosterone stimulation of sodium transport. *Biochim. Biophys. Acta* **135**:74.
13. Farquhar, M. G., and G. E. Palade. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* **26**:263.
14. Fimognari, G. M., G. A. Porter, and I. S. Edelman. 1967. The role of the tricarboxylic acid cycle in the action of aldosterone on sodium transport. *Biochim. Biophys. Acta* **135**:89.
15. Frazier, H. S., E. F. Dempsey, and A. Leaf. 1962. Movement of sodium across the mucosal surface of the isolated toad bladder and its modification by vasopressin. *J. Gen. Physiol.* **45**:529.
16. Katchalski, E., M. Sela, H. I. Silman, and A. Berger. 1964. Polyamino acids as protein models. *In* The Proteins, Vol. II. H. Neurath, editor. Academic Press Inc., New York, p. 405.
17. Katchalsky, A., D. Danon, A. Nevo, and A. de Vries. 1959. Interactions of basic polyelectrolytes with the red blood cell. *Biochim. Biophys. Acta* **33**:120.
18. Kellenberger, E., A. Ryter, and J. Séchaud. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *J. Biophys. Biochem. Cytol.* **4**:671.
19. Keller, A. R. 1963. A histochemical study of the toad urinary bladder. *Anat. Rec.* **147**:367.
20. Koefoed-Johnsen, V., and H. H. Ussing. 1958. The nature of the frog skin potential. *Acta Physiol. Scand.* **42**:298.
21. Kornguth, S. E., M. A. Stahmann, and J. W. Andersen. 1961. Effect of polylysine on the cytology of the Ehrlich ascites tumor cells. *Expl. Cell Res.* **24**:484.
22. Leaf, A., and R. M. Hays. 1962. Permeability of the isolated toad bladder to solutes and its modification by vasopressin. *J. Gen. Physiol.* **45**:921.
23. Lichtenstein, N. S., and A. Leaf. 1965. Effect of amphotericin B on the permeability of the toad bladder. *J. Clin. Invest.* **44**:1328.
24. Lipman, K. M., R. Dodelson, and R. M. Hays. 1966. The surface charge of isolated toad bladder epithelial cells. *J. Gen. Physiol.* **49**:501.
25. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
26. Messier, B., and C. P. Leblond. 1957. Preparation of coated radioautographs by dipping sections in fluid emulsion. *Proc. Soc. Exp. Biol. Med.* **96**:7.
27. Nevo, A., A. de Vries, and A. Katchalsky. 1955. Interaction of basic polyamino acids with the red blood cell. I. Combination of polylysine with single cells. *Biochim. Biophys. Acta* **17**:536.

28. Palade, G. E. 1952. A study of fixation for electron microscopy. *J. Exp. Med.* **95**:285.
29. Peachey, L. D., and H. Rasmussen. 1961. Structure of the toad's urinary bladder as related to its physiology. *J. Biophys. Biochem. Cytol.* **10**:529.
30. Porter, G. A., and I. S. Edelman. 1964. The action of aldosterone and related corticosteroids on sodium transport across the toad bladder. *J. Clin. Invest.* **43**:611.
31. Revel, J. P., and S. Ito. 1967. The surface components of cells. *In* The Specificity of Cell Surfaces. B. D. Davis and L. Warren, editors. p. 211. Prentice-Hall, Inc., Englewood Cliffs, N.J.
32. Richardson, T., J. Hodgett, A. Lindner, and M. A. Stahmann. 1959. Action of polylysine on some ascites tumors in mice. *Proc. Soc. Exp. Biol. Med.* **101**:382.
33. Sharp, G. W. G., C. H. Coggins, N. S. Lichtenstein, and A. Leaf. 1966. Evidence for a mucosal effect of aldosterone on sodium transport in the toad bladder. *J. Clin. Invest.* **45**:1640.
34. Skou, J. C. 1965. Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. *Physiol. Rev.* **45**:596.
35. Trump, B. F., E. A. Smuckler, and E. P. Benditt. 1961. A method for staining epoxy sections for light microscopy. *J. Ultrastruct. Res.* **5**:343.
36. Ussing, H. H. 1960. The frog skin potential. *J. Gen. Physiol.* **43**:135.
37. –, and K. Zerahn. 1951. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23**:110.
38. Venable, J. H., and R. A. Coggeshall. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.