THE EFFECT OF CALCIUM WITHDRAWAL ON THE STRUCTURE AND FUNCTION OF THE TOAD BLADDER

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ABSTRACT

Previous reports have indicated that calcium is necessary to support active sodium transport by the toad bladder, and may be required as well in the action of vasopressin on both toad bladder and frog skin. The structure and function of the toad bladder has been studied in the absence of calcium, and a reinterpretation of the previous findings now appears possible. When calcium is withdrawn from the bathing medium, epithelial cells detach from one another and eventually from their supporting tissue. The short-circuit current (the conventional means of determining active sodium transport) falls to zero, and vasopressin fails to exert its usual effect on short-circuit current and water permeability. However, employing an indirect method for the estimation of sodium transport (oxygen consumption), it is possible to show that vasopressin exerts its usual effect on Q_{O_2} when sodium is present in the bathing medium. Hence, it appears that the epithelial cells maintain active sodium transport when calcium is rigorously excluded from the bathing medium, and continue to respond to vasopressin. The failure of conventional techniques to show this can be attributed to the structural alterations in the epithelial layer in the absence of calcium. These findings may provide a model for the physiologic action of calcium in epithelia such as the renal tubule.

INTRODUCTION

Both frog skin and toad bladder have been of great value in the *in vitro* study of ion transport. The short-circuit technique, developed by Ussing (1), has been used to demonstrate that active sodium transport is responsible for the potential observed across the frog skin; similar studies by Leaf *et al.* (2) have shown the same to be true of the toad bladder. Both tissues show an increase in sodium transport and net water movement in the presence of vasopressin (1–4).

There have been several reports of the effect of calcium withdrawal on ion transport by the toad bladder and frog skin. Bentley (5) has presented evidence that when calcium was withdrawn from the medium bathing the toad bladder, active sodium transport, as measured by the short-circuit technique, was depressed, and failed to show its usual increase in response to vasopressin. He concluded that calcium was necessary to support active sodium transport by this tissue. Curran and associates (6) reported a decrease in net sodium transport across frog skin treated with EDTA; they noted, however, that the decrease could be attributed to a large increase in the passive unidirectional flux of sodium, and that movement of

sodium in the direction of active transport was unimpaired.

We have reexamined the events that take place when calcium is withdrawn from the medium bathing the toad bladder. In many of our experiments, EDTA was added to the bathing medium to ensure the absence of calcium. Our results may be summarized as follows. (*a*) In the absence of calcium, there is a rapid decrease in short-circuit current, electrical potential, and ohmic resistance measured across the tissue. (*b*) There is a simultaneous increase in the permeability of the tissue to inulin, water, chloride, and thiourea. (*c*) These alterations in electrical and permeability properties are partly or wholly the result of detachment of epithelial cells from one another, and eventually from the bladder, as a result of calcium withdrawal. (*d*) Studies of the oxygen consumption of the toad bladder in the absence of calcium indicate that active sodium transport does in fact increase in its usual fashion in response to vasopressin. (*e*) Calcium plays an important role in maintaining the adherence of epithelial cells to one another, and, by regulation of the precise extent of cell adhesion, may thereby take part in the regulation of ion and water movement across the tissue under physiological conditions. The results of our experiments on the toad bladder are similar to those recently reported by Sedar and Forte for frog gastric mucosa (7). Hays and Singer (25) and Peachey (26) have reported the detachment of toad bladder epithelial cells in the absence of calcium; Malamed (9) has also described these cells.

MATERIALS AND METHODS

Materials

Adult female toads (*Bufo marinus*), supplied by the National Reagents Company, Bridgeport, Connecticut, were used in the studies to be reported. The toads were kept on moist earth, and were not fed. The C^{14}-thiourea, tritiated water, and C^{14}-inulin used in these studies were supplied by the New England Nuclear Corporation, Boston, Massachusetts; the chloride-36 was supplied by Tracerlab, Inc., Waltham, Massachusetts. The calcium-free amphibian Ringer's solution used in the studies had the following composition: NaCl 112, $NaHCO_3$ 2.4, and KCl 3.5 mEq per liter. The pH was 8.1. The distilled water used in making up the Ringer's solution was further deionized by running it through a Bantam BD-1 demineralizing cartridge (Barnstead Still and Sterilizer Company, Boston, Massachusetts). The calcium-containing Ringer's had 1 mmole $CaCl_2$ per liter in addition to the above components.

Measurement of Short-Circuit Current and Resistance

Net sodium movement was determined by the short-circuit method of Ussing and Zerahn (1). Half of a bilobed bladder was removed from a doubly pithed toad and dipped briefly 3 times in 30 ml of either calcium-free or calcium-containing Ringer's solution and then mounted in a Lucite chamber. Fifteen milliliters of the appropriate Ringer's solution were placed in each chamber half. Measurements of short circuit current and resistance were started immediately after the chamber was filled. Resistance was measured by increasing the observed short-circuit current by 100 microamperes and observing the resulting deflection of the millivoltmeter. In other experiments, a bladder half was mounted as a bag on a glass bung, and the bag suspended in a beaker containing the appropriate Ringer's solution. Short-circuit current and resistance were monitored with KCl-agar bridges placed inside and outside the bag, as described by Bentley (5).

Permeability Studies

The permeability of the toad bladder to a variety of isotopically labeled substances was determined by placing the isotope in one half of the Lucite chamber and measuring its rate of appearance in the other. In experiments in which the bladder was mounted as a bag on a glass bung, isotope was added to the small volume (2 to 4 ml) of Ringer's solution within the bag. The rate of appearance of isotope in the outside solution was determined as in the chamber experiments; flux rates were expressed as permeability coefficients (K_{trans}), as previously described (4). Samples withdrawn from the inside and outside solutions were pipetted into vials containing 15 ml of scintillation solution (0.05 gm *p*-bis(2-(5-phenyloxazolyl)) benzene; 7 gm 2-5-diphenyloxazole; and 50 gm naphthalene) made up to a liter with *p*-dioxane. The final water content was 1.0 ml per 15 ml of scintillation mixture. The vials were placed in a Packard Liquid Scintillation Counter (Packard Instrument Company, La Grange, Illinois). It was found that the level of counts in the samples containing C^{14}-inulin decreased with time, apparently as a result of the polymer's being absorbed on the glass of the counting vials (8). Three grams of Cab-O-Sil (silicon dioxide) furnished by Godfrey L. Cabot, Inc., were added to every 100 ml of scintillation solution, and no further difficulty was encountered.

Studies of Oxygen Consumption

WHOLE TISSUES: In studies of oxygen consumption, half bladders were incubated in the appropriate bathing medium (see Results), then cut into

small pieces, and placed in 6-ml Warburg flasks with 10 per cent KOH in the center well. The medium used to suspend the bladder pieces was amphibian Ringer's buffered with bicarbonate (2.4 mEq per liter, pH 8.1), or phosphate (3 mmoles per liter, pH 7.5). The flasks were placed in a G. M. E.-Lardy Warburg apparatus, Model WB4 (Gilson Medical Electronics, Middleton, Wisconsin), and after a 15-minute equilibration period, the manometers were closed off and oxygen consumption (Q_{O_2}) was recorded. After this procedure, the bladders were removed from the flasks, carefully blotted with Whatman no. 54 filter paper, and placed in an oven at 95°C overnight. The dry weight of the tissue was then determined, and oxygen consumption was calculated as microliters of oxygen consumed per milligram dry weight per hour.

ISOLATED CELL SUSPENSIONS: It was possible to obtain large numbers of isolated epithelial cells, completely detached from the supporting tissues of the toad bladder, by the following method. Six to eight bladder halves were incubated in 400 to 600 ml of calcium-free Ringer's solution which usually contained EDTA, 1.5 mmoles per liter. The pH of the Ringer's solution was adjusted to 8.0. After 1 hour of incubation, each bladder half was picked up with forceps and the neck closed off with a hemostat. The bag thus formed was gently massaged between thumb and forefinger; the bag was then cut open and the contents were collected in a centrifuge tube. The solution obtained in this manner was cloudy, and microscopic examination showed it to contain numerous isolated epithelial cells, mostly single cells, but with small and large clusters as well. A concentrated suspension of cells was prepared for studies in the Warburg apparatus by centrifuging the suspension at 2000 RPM for 15 to 30 seconds, and pipetting 0.7 ml of the loose button of sedimented cells into a Warburg flask. Following the determination of oxygen consumption, the cell suspension was carefully removed from the flask and centrifuged for 10 minutes, the supernatant was removed, and the cells were washed onto a cup of aluminum foil with distilled water. The dry weight was determined after heating overnight in an oven at 95°C.

Electron Microscopy

Whole tissues and isolated cells were fixed in 1 per cent osmium tetroxide in calcium-containing bicarbonate-buffered amphibian Ringer's solution for 1 hour and prepared for electron microscopy by a method previously described (9). The embedding medium was Epon 812 of medium hardness (10). Sections of the samples were stained in 0.5 per cent uranyl acetate in 50 per cent ethanol (11). The samples were examined in the Philips EM 75B and the RCA EMU 3E microscopes.

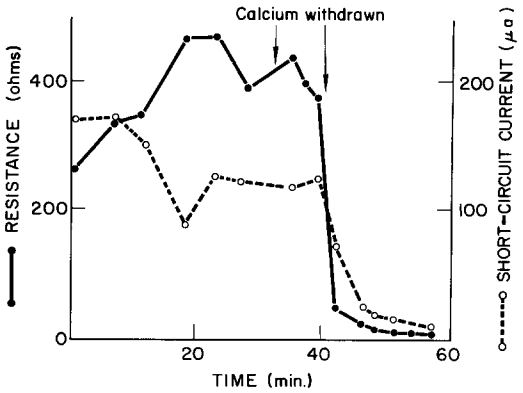


FIGURE 1 The effect of calcium withdrawal on short-circuit current and resistance. At the point indicated by the first arrow, the serosal bathing medium was changed from calcium-containing to calcium-free Ringer's solution; a fresh calcium-free bath was used at the point indicated by the second arrow.

RESULTS

Effects of Calcium Withdrawal on Resistance and Short-Circuit Current

In the first series of experiments, the effect of calcium withdrawal on ohmic resistance and short-circuit current was determined. The short-circuit current has been shown to be identical with the net or active sodium transport of this tissue when Ringer's solution of the same composition bathes each surface (2). Net sodium transport proceeds from the luminal (mucosal) surface to the nutrient (serosal) surface of the epithelial cells. The ohmic resistance may be interpreted as a measure of the ease with which ions penetrate the tissue. The higher the resistance, the lower the ionic permeability.

Fig. 1 shows the resistance and short-circuit current of a half bladder mounted on a glass bung, with calcium-free Ringer's solution bathing the inside (luminal) surface, and calcium-containing Ringer's solution bathing the outside (nutrient) surface. The usual 400- to 500-ohm resistance and 100- to 200-μa short-circuit current are shown. As indicated by the arrows, the outside bath was changed to calcium-free Ringer's solution. This was done twice, to leave as little calcium as possible in the medium. There was a rapid fall in both the resistance and the short-circuit current, and both eventually approached zero. A similar but more rapid decrease in resist-

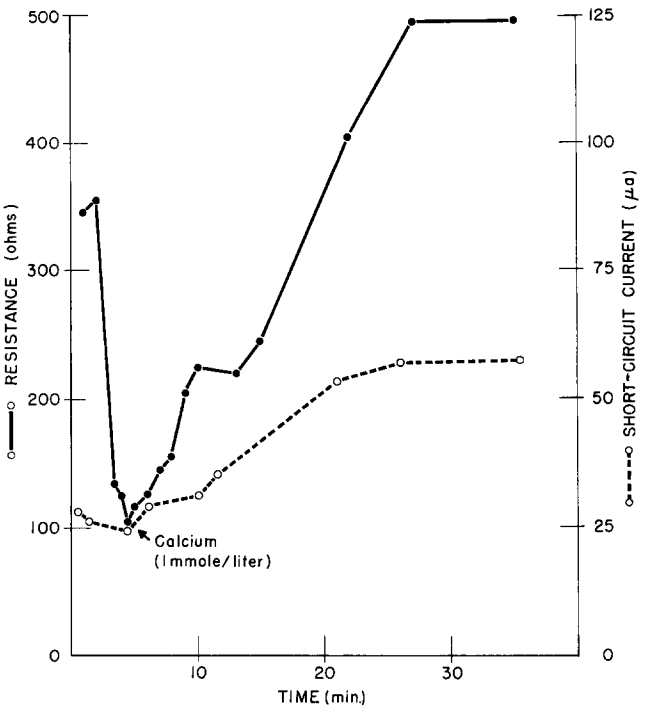


FIGURE 2 The effect of the readdition of 1 mmole calcium per liter to the medium bathing the mucosal and serosal surfaces of the bladder on short-circuit current and resistance. Both surfaces of the bladder were initially bathed in calcium-free Ringer's solution.

ance and short-circuit current was seen when EDTA was present in the bathing medium. When, in other experiments, the short-circuit current reached zero, the addition of vasopressin to the bathing medium was without its usual effect, and the short-circuit current remained zero.

When the bladder was rinsed in calcium-free Ringer's solution and placed in a Lucite chamber filled with calcium-free Ringer's, the ohmic resistance again fell sharply; however, if calcium was readded to the bathing medium before the resistance reached zero, resistance rose rapidly to normal levels, and the short-circuit current remained high (Fig. 2).

Effect of Calcium Withdrawal on Permeability

Table I shows the permeability of the toad bladder to water, thiourea, and chloride when calcium was present in the bathing medium, and in the absence of calcium. The data were obtained using Lucite chambers, with calcium-containing or calcium-free Ringer's solution bathing the bladder. In the absence of calcium, there was a large increase in the permeability of the bladder to all these substances. The characteristic increase in the permeability of the bladder to water following vasopressin was not seen in the absence of calcium.

TABLE I

Effect of Calcium Withdrawal on the Permeability of the Toad Bladder to Water, Thiourea, and Chloride

	K_{trans} (cm $sec^{-1} \times 10^7$)			
	calcium present*		Calcium absent	
Water	944	1580‡	2650	1900‡
Thiourea	14	—	784	—
Chloride	13	—	1200	—

* Values from Leaf and Hays (23).
‡ K_{trans} following vasopressin.

The permeability of the bladder to C^{14}-inulin was also determined (Fig. 3). The experiment is that of Fig. 1; data for the K_{trans} of inulin are now shown. In the presence of calcium, the bladder was virtually impermeable to a molecule as large as inulin. The small amount of inulin that did move across the bladder probably did so by moving between rather than through the epithelial cells. When calcium was withdrawn from the bathing medium, the permeability of the bladder to inulin rose rapidly to a level of 60 times its original value.

Effect of Calcium Withdrawal on the Structure of the Toad Bladder

Fig. 4 shows a portion of the epithelial lining of the lumen of the untreated, control toad bladder. Several detailed accounts of its structure have already appeared (12–14). The largest part of the field is occupied by a mitochondrion-rich cell. The adjoining cells seen in Fig. 4 are granular cells, according to the classification of Choi (13). In other preparations, basal cells and mucus cells have been observed. The numerous microvilli are filled with an electron-opaque, apparently fibrous material which in several instances extends into the cytoplasm below the base of the microvilli. Frequently, a fuzzy covering on the luminal surfaces of the microvilli is also encountered. The morphological features of greatest pertinence to the present studies are the two sorts of relationships between the lateral surfaces of adjacent epithelial cells. In many sites, pairs of plasma membranes are thrown into convoluted folds of great complexity. Another kind of relationship is the juxtaluminal junctional complex, which at low magnification appears to conform to the generalized tripartite scheme advanced recently by Farquhar and Palade (15). One component of the complex, the desmosome, frequently appears singly.

In calcium-free medium (in the presence of EDTA), the epithelial cells detach from one another (Fig. 5). This dissociation is accompanied by the disruption of both kinds of cell attachment discussed above. Fig. 5 shows a bladder which was exposed to calcium-free Ringer's solution plus EDTA for 20 minutes. A group of partly separated epithelial cells, whose adjacent plasma membranes are "unrolled" in at least two sites, is seen. A junctional complex is shown with a separation in the intermediate (zonula adhaerens) region. Immediately above and below a few isolated desmosomes, other separations occur. These observations lend support to current concepts of the varying importance of each of these side-to-side specializations of the plasma membrane in keeping the cells attached (7, 15).

Cell dissociation is complete when the epithelium is gently massaged in a calcium-free medium. Fig. 6 shows a group of free cells which had been treated in this fashion. Their morphology has been altered in the process of detachment in at least three ways: (*a*) they are now rounded in shape, (*b*) they possess numerous electron-transparent vacuoles, and (*c*) in some but not all cells, mitochondrial swelling is extensive. However, different cell types are still clearly distinguishable, and microvilli are abundantly apparent.

Oxygen Consumption in the Absence of Calcium

It is clear that the withdrawal of calcium from the bathing medium results in a rapid and extensive change in the organization of epithelial cells of the toad bladder. The changes in permeability and electrical properties can be attributed at least in part to the separation of epithelial cells, with the development of large aqueous channels

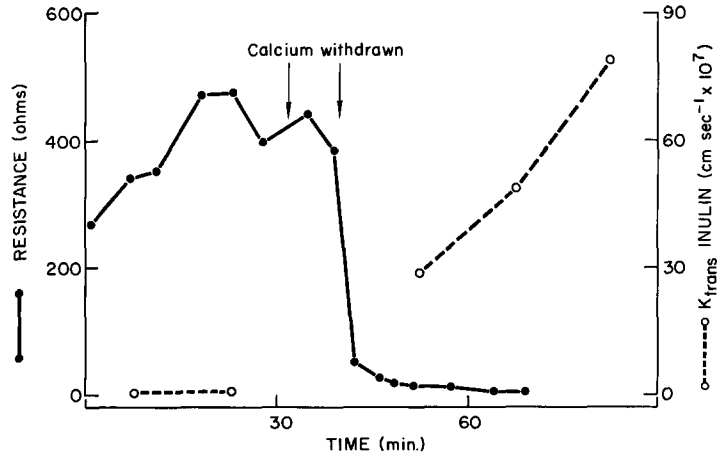


FIGURE 3 The effect of calcium withdrawal on the permeability of the toad bladder to inulin. Two control periods are shown, and, following calcium withdrawal, three additional periods. The experiment is the same as that shown in Fig. 1.

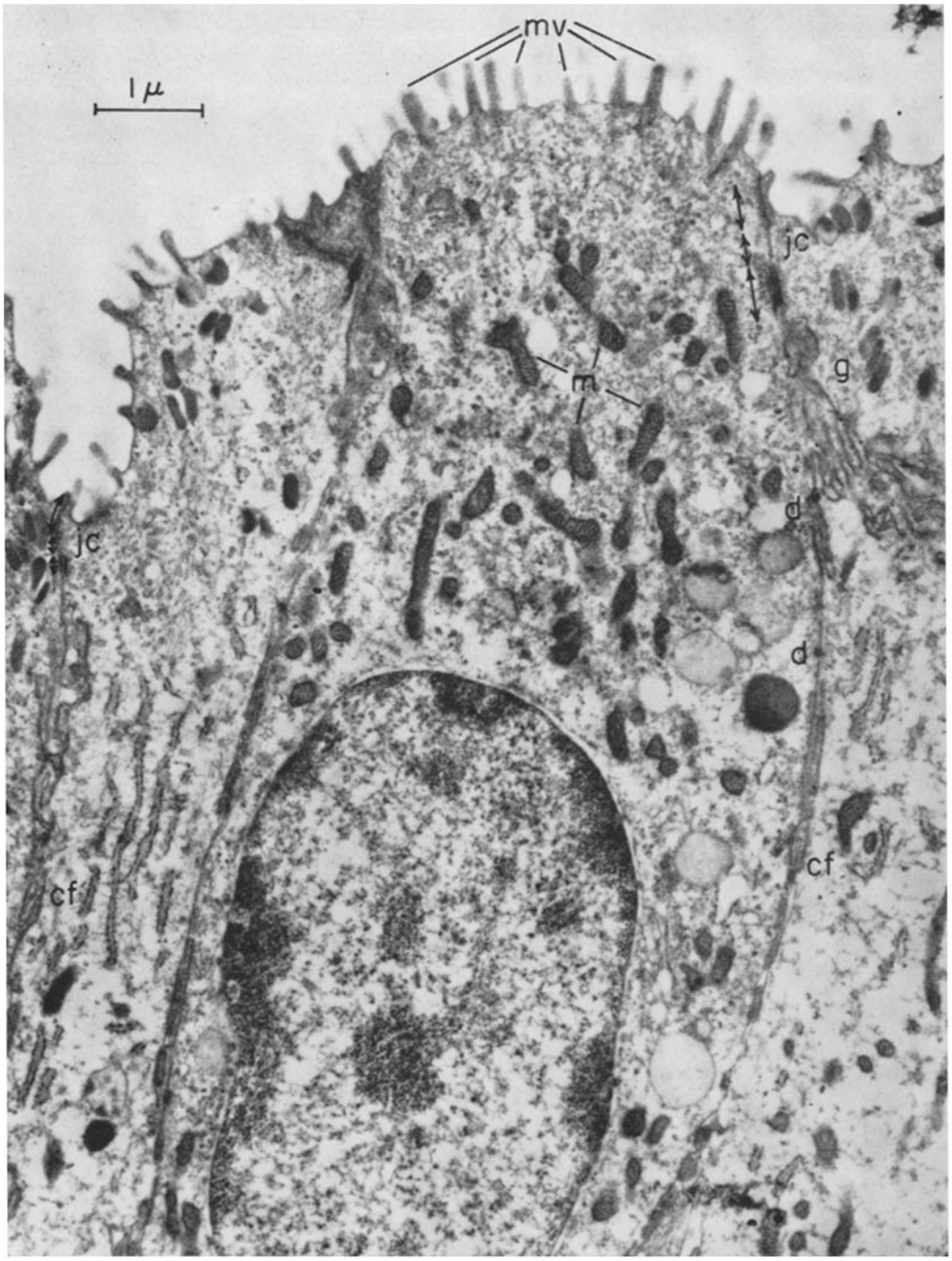


FIGURE 4 Untreated, control epithelial cells lining the luminal surface of the urinary bladder of the toad. Two characteristic specializations of the lateral plasma membranes of adjacent cells are shown: convoluted folds (*cf*) and juxtaluminal junctional complexes (*jc*); in each of the latter, three linear regions may be distinguished (double-headed arrows). Other attachments, occurring singly, are presumably desmosomes (*d*), and are similar to or identical with the component of the junctional complex which is farthest from the lumen. A mitochondrion-rich cell occupies most of the field; the others are granular cells, except the one in the lower right corner, which may be a basal cell. *mv*, microvilli; *g*, granules; *m*, mitochondria. × 15,500.

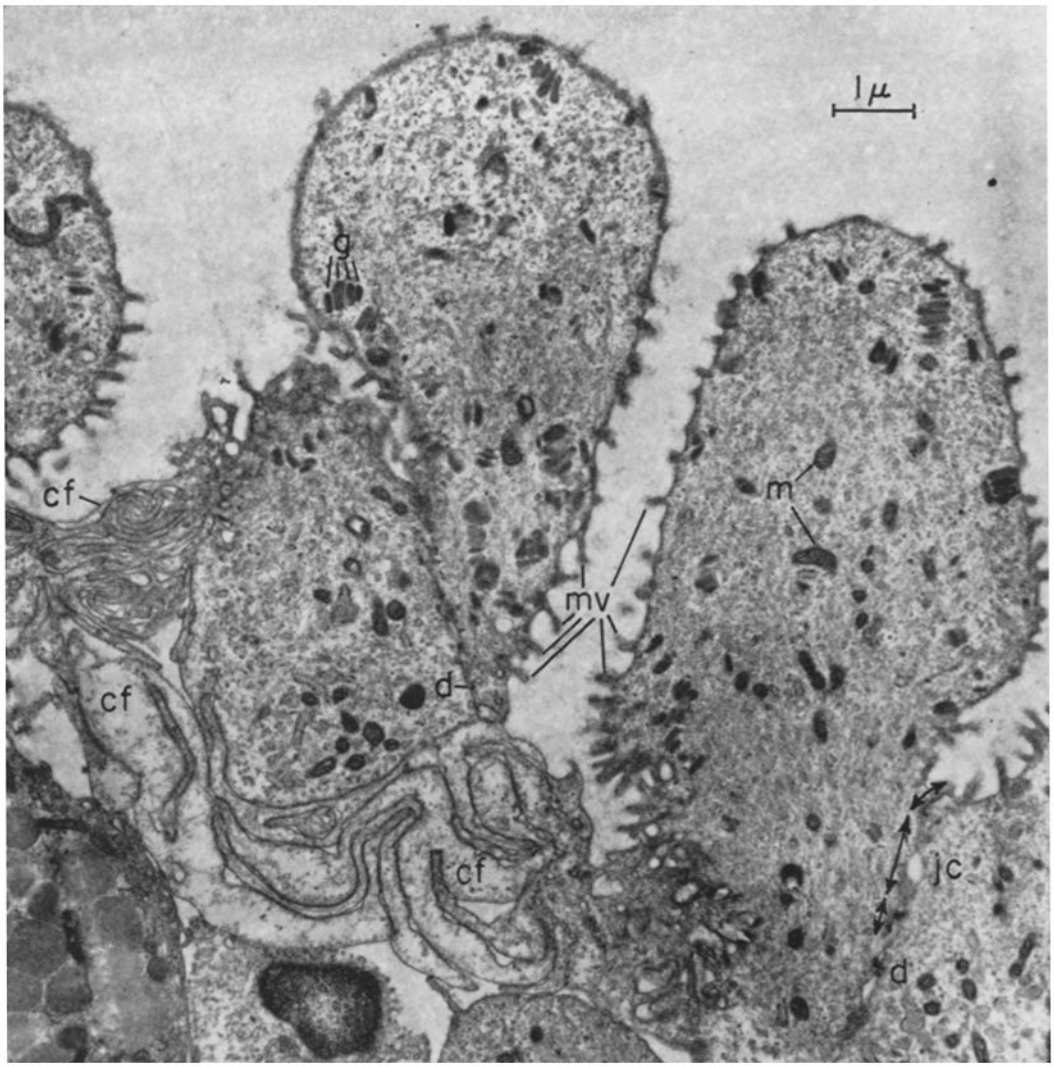


FIGURE 5 Dissociating epithelial cells in calcium-free medium in the presence of EDTA. Both kinds of lateral cell attachments are disrupted. "Unrolling" of convoluted folds (*cf*) is apparent, as is the separation of a tripartite junctional complex in its intermediate region (*jc*). Similarly, other separations between adjacent plasma membranes appear above and below desmosomes (*d*). A mucus cell occupies the lower left corner of the field. *mv*, microvilli; *g*, granules; *m*, mitochondria. × 11,500.

between them. Under these conditions, the electrical resistance and potential measured across the tissue would fall to zero, and it would not be possible to measure a "short-circuit current." It appeared conceivable, then, that the separated epithelial cells could maintain their ability to carry on active sodium transport and respond to vasopressin without our being able to confirm this by the usual techniques of determining short-circuit current or measuring permeability. Net water movement following vasopressin in the presence of an osmotic gradient would also be expected to approach zero, since the osmotic gradient would be rapidly dissipated under the experimental conditions.

In an attempt to determine whether active sodium transport and response to vasopressin were indeed maintained, the oxygen consumption of

whole tissues and isolated epithelial cells was studied. The experiments were based on the observations of Zerahn (16) and Leaf *et al.* (17) that a significant portion of the total oxygen consumption of this tissue is accounted for by active sodium transport. When vasopressin is added to sodium-containing Ringer's solution bathing the bladder, there is an increase in Q_{O_2} (18). This typical response of Q_{O_2} to vasopressin in the presence of both sodium and calcium is shown in part A of Table II. In these experiments, portions of paired bladder halves were incubated in calcium-containing Ringer's solution for 45 to 90 minutes, cut into small pieces, and placed in Warburg flasks. Parke-Davis vasopressin, 40 milliunits, was placed in the side arm of one flask. The pH of the vasopressin solution was made identical with that of the Ringer's solution in the Warburg flasks with KOH. Ringer's solution was present in the side arm of the control flask in the first two experiments. In the last four experiments a solution containing only the preservative present in the Parke-Davis vials (4.5 per cent chloretone in distilled water brought to pH 3 with glacial acetic acid, then adjusted to the pH of the Ringer's solution with KOH) was placed in the side arm of the control flask. After a suitable base line was obtained, vasopressin and the control solution were tipped into the flask. The mean increase in the Q_{O_2} of the hormone-treated tissue as compared with the untreated control was 0.25 ± 0.05 (SE) μl/mg dry wt/hr. Three additional experiments were carried out in phosphate-buffered Ringer's solution which contained 0.1 mEq calcium per liter, an amount of calcium adequate to prevent cell separation (22). Q_{O_2} increased in all three experiments by a mean value of 0.32 μl/mg dry wt/hr. following vasopressin.

In our second series of experiments, we attempted to determine whether, in the absence of calcium, oxygen consumption was increased by vasopressin, and, further, whether this response was dependent on the presence of sodium in the bathing medium. In the first and earliest group of these experiments the sodium-containing Ringer's had 22 mEq sodium per liter rather than the usual 112 mEq per liter; choline replaced the rest of the sodium. Bicarbonate buffer was used. The sodium concentration was made low because it was thought that in the absence of calcium the permeability of the epithelial cells to sodium might be significantly increased. The addition of hormone, which apparently increases the permeability of the luminal surface of the epithelial cells to sodium, would then have no further effect on sodium transport if the pump was already saturated.

The procedure in these experiments was as follows. Paired half bladders were dipped for 1 minute in three 25-ml vials of calcium-free Ringer's solution containing 22 mEq sodium chloride per liter and 92 mEq choline chloride per liter. The bladder halves were then incubated in 100 ml of Ringer's of the same composition for 20 minutes and in 50 ml of the same Ringer's containing 1.5 mmoles per liter disodium EDTA for an additional 20 minutes. Determination of the ohmic resistance of two representative bladder halves at this point in the procedure gave values of less than 20 ohms in both cases. The bladder halves were then cut into three pieces, and the pieces were washed with approximately 20 ml of sodium-choline Ringer's and placed in Warburg flasks. Vasopressin or Ringer's solution was placed in the side arms of the paired flasks. In the last three experiments, the chloretone–acetic acid solution was used in the side arm of the control flask. The results in these three experiments were no different

FIGURE 6 Free epithelial cells after massage in calcium-free medium. A mitochondrion-rich cell is seen in the upper right; adjacent to it is a cell containing many dense droplets and a few relatively pale ones, near the nucleus. This is probably a mucus cell, but the possibility exists that the more numerous dark droplets are lipid. The four cells below are granular cells whose nuclei have become indented. Microvilli (*mv*) appear on most cells. Many of the microvilli retain their fuzzy covering (opposite the curved and straight bars). Morphological alterations of the epithelium from the intact state (see Fig. 4) are demonstrated by the rounded shape of the cells, by the presence of numerous electron-transparent vacuoles (*v*), and by extensive mitochondrial swelling in some cells (*m(sw)*), but not in others (*m*). *g*, granules. × 4000.

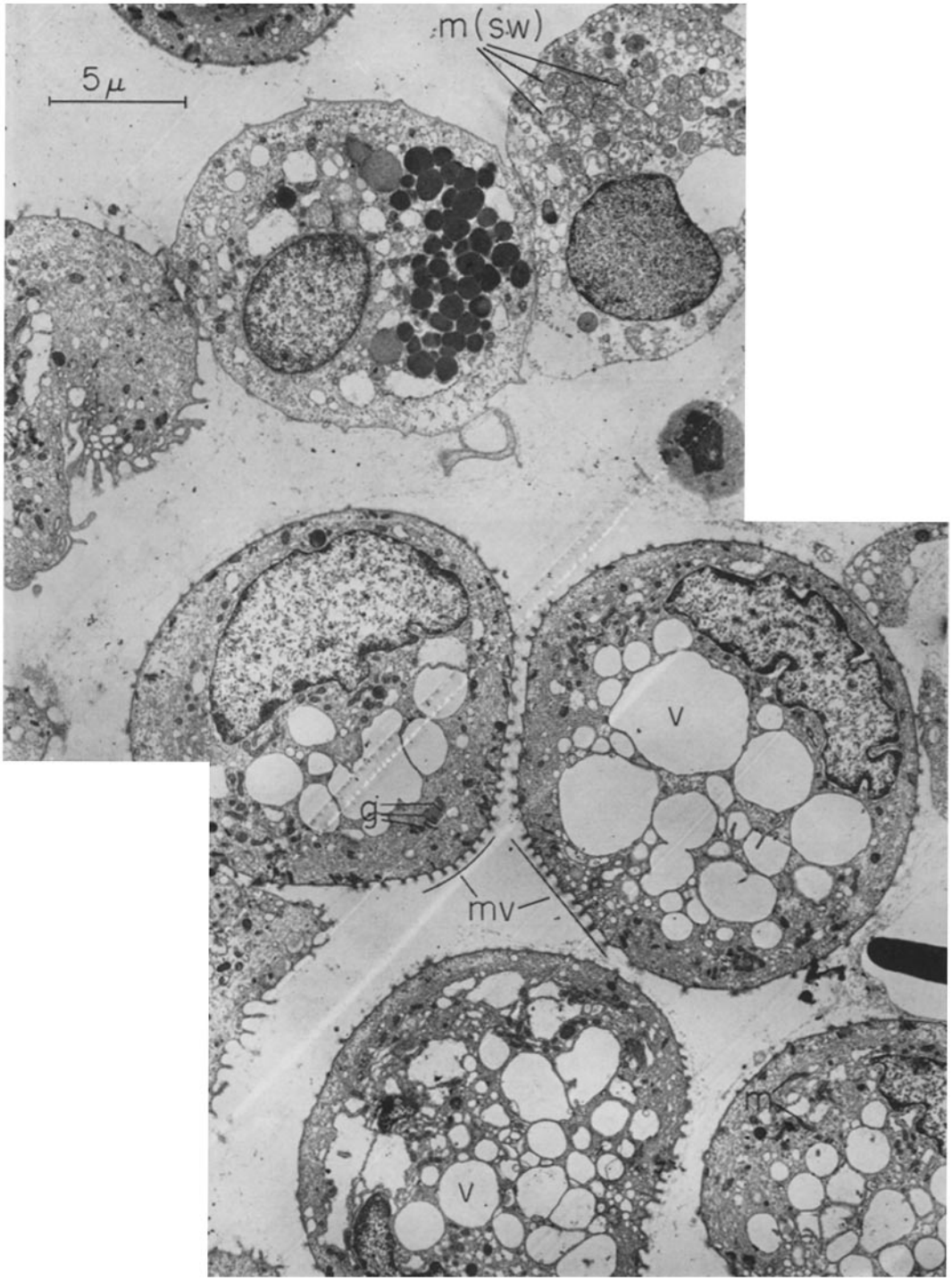


FIGURE 6

TABLE II

Effect of Vasopressin on the Oxygen Consumption of Intact Toad Bladder in the Presence of Calcium

	Q_{O_2} (μl/mg dry wt/hr.): Period*	
	I	II
A. Bicarbonate buffer (6)‡		
Control	1.58	1.46
Vasopressin§	1.64	1.77
	$\Delta = 0.25 \pm 0.05$; $p < 0.01$	
B. Phosphate buffer (3)		
Control	1.49	1.47
Vasopressin	1.48	1.78
	$\Delta = 0.32 \pm 0.13$	

* Periods in this and in subsequent experiments were one-half hour.

‡ Numbers in parentheses refer to the number of paired experiments.

§ Vasopressin added between the first and second periods in this and in subsequent experiments.

TABLE III

Oxygen Consumption of the Toad Bladder in the Absence of Calcium and in the Presence of Sodium

	Q_{O_2} (μl/mg dry wt/hr.): Period	
	I	II
A. Bicarbonate buffer (10)		
Control	1.57	1.34
Vasopressin	1.61	1.61
	$\Delta = 0.23 \pm 0.04$; $p < 0.001$	
B. Phosphate buffer (9)		
Control	1.83	1.67
Vasopressin	1.78	1.89
	$\Delta = 0.27 \pm 0.07$; $p < 0.01$	
C. Scraped bladders, phosphate buffer (3)		
Control	0.33	0.16
Vasopressin	0.58	0.07

22 mEq sodium per liter present in medium of group A; 112 mEq sodium per liter in medium of groups B and C.

from those in the previous seven, all showing an effect of vasopressin on Q_{O_2}. The results of the ten paired experiments are shown in part A of Table III. The Q_{O_2} of the control bladders decreased significantly between periods I and II, whereas the vasopressin-treated bladders showed no change. The mean difference in Q_{O_2} between control and hormone-treated bladders was 0.23 ± 0.04 (SE) μl/mg dry wt/hr.

In nine later experiments, paired half bladders were incubated as in previous experiments, but were then dipped for 2 minutes in two 25-ml volumes of phosphate-buffered calcium-free Ringer's and placed in Warburg flasks containing phosphate rather than bicarbonate buffer. The sodium concentration was 112 mEq per liter. In six of these experiments, Parke-Davis vasopressin or Ringer's solution was placed in the side arms of the paired flasks. In three, 14 milliunits of synthetic arginine-8 vasopressin (Sandoz) was em-

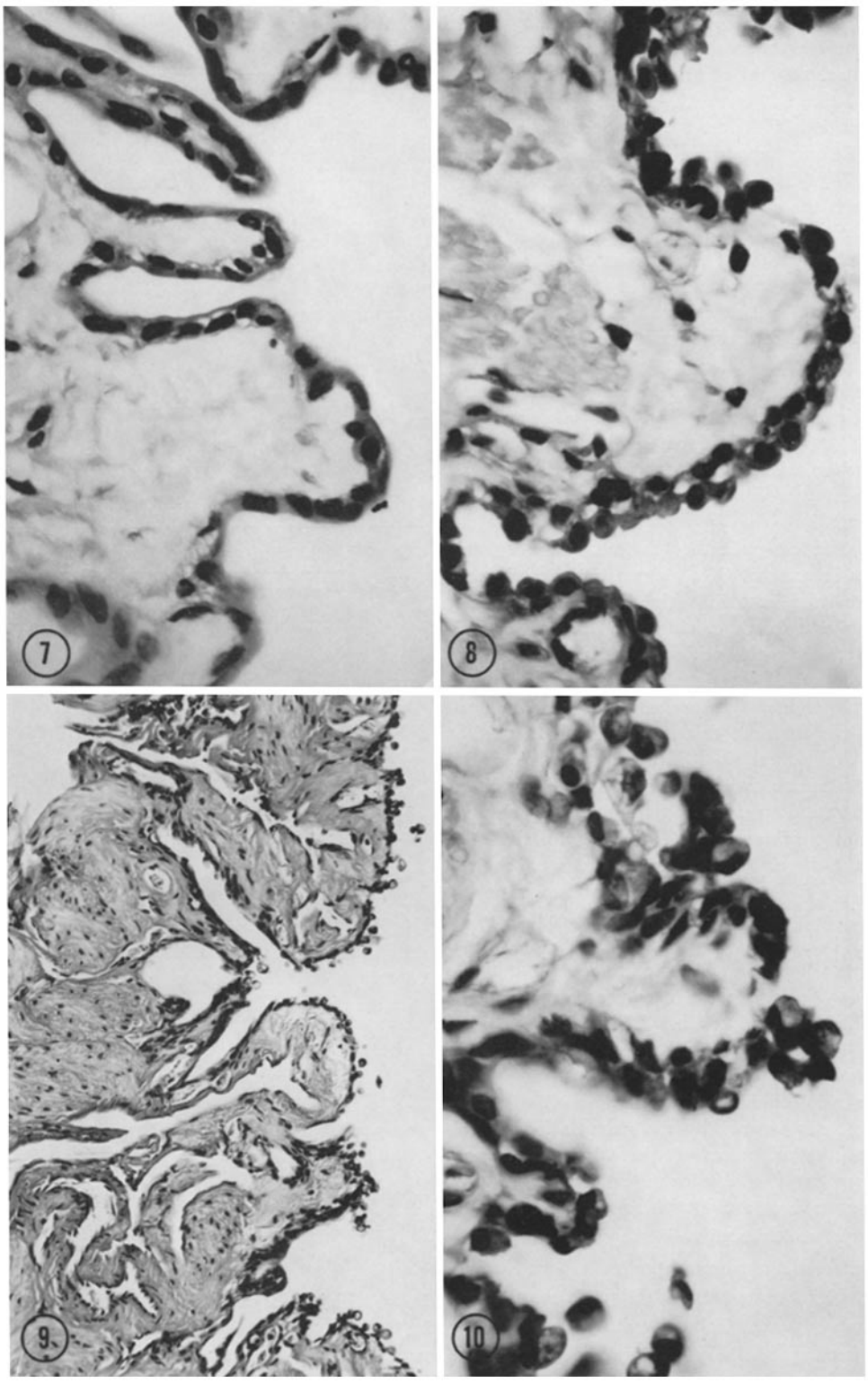
FIGURE 7 Hematoxylin- and eosin-stained section of normal toad bladder in calcium-containing Ringer's solution. × 420.

FIGURE 8 Section of bladder after 20 minutes of incubation in calcium-free Ringer's, 20 minutes in calcium-free Ringer's containing EDTA, and two dips in phosphate-buffered calcium-free Ringer's. The epithelial layer shows some signs of early disorganization with rounding of epithelial cells; in other areas of the same bladder the epithelial layer is grossly indistinguishable from (Fig. 7). × 420.

After this point in the procedure, the bladders are placed in Warburg flasks.

FIGURE 9 Section of bladder after 2 hours of shaking in the Warburg apparatus. Abundant epithelial cells are present, many still firmly attached to the bladder, and many in various stages of detachment. × 125.

FIGURE 10 Higher power view of the section shown in Fig. 9. × 420.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ployed. Oxygen consumption increased comparably following the Parke-Davis or the synthetic vasopressin; the mean difference between hormone-treated and control bladders was 0.27 ± 0.07 (Table III, part B). Microscopic examination of a portion of bladder after 2 hours of shaking in the Warburg apparatus showed that abundant epithelial cells remained attached to the bladder, although the epithelial cell layer was greatly disorganized (Figs. 7 to 10).

To make sure that the increase in Q_{O_2} could be attributed to the epithelial cells, and not to some element of the bladder such as smooth muscle, the epithelial cells were scraped from the bladder with a coverslip. The scraped bladders, free of epithelial cells, were then incubated in calcium-free Ringer's as in the previous experiments, and their Q_{O_2} was determined in phosphate-buffered Ringer's solution. The Q_{O_2} of this preparation was low, and was unaffected by vasopressin (Table III, part C).

To determine whether sodium was necessary for the increase in Q_{O_2}, eight paired experiments were carried out, in which bladders were incubated as before in calcium-free EDTA Ringer's; however, choline chloride replaced all the sodium chloride in the Ringer's solution. Although 3 mEq sodium per liter was present in the medium as disodium versenate during the incubation with EDTA, the final rinse of the bladder pieces in calcium-free choline Ringer's reduced this sodium concentration considerably. Choline Ringer's was used in the side arms of all the control flasks. The results of the eight paired experiments are shown in Table IV. The increase in Q_{O_2} of the hormone-treated tissues as compared with the controls was 0.06 ± 0.03 (SE) μl/mg dry wt/hr. The slight increase is of questionable significance ($p < 0.1$). Four additional experiments were carried out in phosphate-buffered choline Ringer's (Table IV, part B). There was no increase in Q_{O_2} following vasopressin.

Our results indicate that the action of vasopressin, as measured by the increase in oxygen consumption of pieces of whole bladder, is not impaired by the absence of calcium from the bathing medium, and that this action is dependent on the presence of sodium in the bathing medium.

Several series of paired experiments were carried out in bicarbonate-buffered Ringer's solution to determine whether isolated epithelial cells, massaged free from the bladder, showed an increase in Q_{O_2} in the presence of vasopressin. Cell suspensions were prepared as outlined in the Methods section, and placed in Warburg flasks. Vasopressin was added either before or after massage to one group of cells, and chloretone–acetic acid to the control cells. The results of three such studies are shown in Table V. The mean Q_{O_2} of vasopressin-treated cells consistently exceeded that of control cells; however, the results were not statistically significant in all cases. The large standard errors probably reflect the difficulties in measuring the relatively low total oxygen consumption of the cell suspensions as compared with those of the whole tissues, as well as inaccuracies in determining the dry weights of the cells.

TABLE IV

Oxygen Consumption of the Toad Bladder in the Absence of Both Calcium and Sodium

	Q_{O_2} (μl/mg dry wt/hr.): Period	
	I	II
A. Bicarbonate buffer (8)		
Control	1.00	0.91
Vasopressin	1.00	0.97
	$\Delta = 0.06 \pm 0.03$; $p < 0.1$	
B. Phosphate buffer (4)		
Control	0.77	0.77
Vasopressin	0.79	0.78
	$\Delta = -0.01 \pm 0.03$	

TABLE V

Oxygen Consumption of Isolated Epithelial Cells in the Absence of Calcium; Bicarbonate Buffer Only

No. paired experiments	Q_{O_2} (μl mg dry wt/hr.)			
	Vasopressin	Contol	Δ	p
9*	2.38	1.55	0.83 ± 0.21	<0.01
6	2.38	2.10	0.28 ± 0.13	<0.1
7	2.54	2.27	0.27 ± 0.16	<0.2

* 1.5 mmoles EDTA per liter present in bathing medium of the first two series of experiments, but absent in the third.

DISCUSSION

When calcium is withdrawn from the medium bathing the toad bladder, there are a number of rapid alterations in the electrical and permea-

bility properties of the tissue. Short-circuit current, resistance, and potential are decreased and eventually reach zero. The permeability of the bladder to a large number of solutes, including inulin, is greatly increased. The usual effects of vasopressin on short-circuit current and unidirectional water flux are unobtainable. Net water movement in the presence of vasopressin and an osmotic gradient has been reported to be significantly decreased (19).

These alterations in the function of the tissue can be partly, perhaps completely, explained by the morphologic changes that take place in the absence of calcium. The epithelial cells, which are ordinarily tightly joined to one another and to the basement membrane, become separated, and wide aqueous channels open between them. One would predict the changes in electrical and permeability properties that do, in fact, occur.

The importance of calcium in cell adhesiveness has been recognized for many years, but the exact mechanism is not known. Steinberg (20) has suggested that calcium may serve as a direct link between acid groups on adjoining cell surfaces; Curtis (21), on the other hand, believes that calcium may decrease the negative surface charge of the cell and, by thus decreasing the repulsive forces between cells, allow their closer approximation, where van der Waals–London forces may promote adhesion. The present studies add little to our understanding of the mechanism of cell adhesion; however, two points can be made. First, whatever the nature of the calcium bond, it appears to break down readily when calcium is withdrawn from the medium; EDTA is not necessary for cell separation to take place. Second, we have found that simply possessing a double valence does not qualify an ion to promote cell adhesion. Manganese and strontium are able to prevent the rapid fall in resistance that takes place when calcium is withdrawn from the medium; magnesium, cobalt, zinc, and barium are not (22).

At what region on the cell surface calcium acts to promote adhesion is not clear. Studies by Choi (13) have demonstrated a region close to the lumen where adjoining cell membranes become so tightly apposed as to appear fused. This is characteristic of a number of epithelia recently studied by Farquhar and Palade (15). The intercellular distance in this region may be small enough to allow calcium to participate in a direct bond between acid surface groups of adjoining cells.

Despite the extensive change in the structure of the bladder that follows calcium withdrawal, active sodium transport appears to be maintained by the epithelial cells. This conclusion is based on the characteristic increase in Q_{O_2} that takes place in the presence of vasopressin, and the demonstration that the increase in Q_{O_2} depends on the presence of sodium in the bathing medium. The results of our studies on epithelial cells massaged free from the bladder are equivocal, and may be so as a result of alterations in the cells following massage, or of the relative inability of the present Warburg technique to measure small absolute changes in Q_{O_2}.

Our concept of the site of action of vasopressin is based largely on studies by Leaf and associates (23, 24). The hormone appears to increase the permeability of the luminal surface of the epithelial cell to sodium (as well as to urea and water). As a result of the increased intracellular concentration of sodium, there is an increase in the activity of the sodium pump, and sodium leaves the cell at an increased rate, across the basal or lateral surface or both. Our studies would suggest that this sequence of events can take place even where the lateral borders of the cells are widely separated. It is possible, of course, that vasopressin acts only in sites on the tissue where epithelial cells are still tightly apposed; some sections of the calcium-depleted bladder show closer approximation than do others (Fig. 9). Nevertheless, the almost identical increment in oxygen consumption of vasopressin-treated bladders in the presence of calcium (Table II) and of EDTA (Table III) suggests that vasopressin maintains its full effect when intercellular attachments are being broken down. It therefore appears unlikely that intercellular mechanisms play a critical role either in the ability of the epithelial cells to maintain active transport, or in the action of vasopressin. However, the *rate* of net transport may be influenced by the spatial relationships between adjoining cells. For example, the ohmic resistance across which sodium is pumped may vary with small changes in intercellular spacing; furthermore, the passive movement of sodium would be expected to increase as cells became more widely separated. Calcium, then, may exert some of its known effects on ion transport by bringing about small changes in the nature and extent of intercellular binding. The present experiments do not allow a choice between this possibility and others; for example, it

seems entirely possible that calcium modifies the luminal surface of the epithelial cell in such a way as to control the entry of ions and water into the cell.

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