J. Membrane Biol. 6, 108–126 (1971)

Measurement of the Composition of Epithelial Cells from the Toad Urinary Bladder

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Received 9 March 1971

Summary. Two methods are described by which epithelial cells from toad urinary bladders can be obtained for analysis of their intracellular water and electrolyte contents. In the first, a method similar to that described in 1968 by J. T. Gatzy and W. O. Berndt, sheets of epithelial cells are scraped from bladders after incubation in sodium Ringer's and collagenase (400 mg/liter). The scraped cells were incubated under various conditions and their composition subsequently determined. Oxygen consumption was also measured. In the second method, epithelial cells were scraped from hemibladders removed from chambers. These cells were then analyzed without further incubation. The morphology of epithelial cells obtained by each method is illustrated. Both methods yield similar results and evidence is provided that the derived intracellular values obtained truly reflect the composition of the epithelial cells.

The urinary bladder of the toad is one of the epithelial membranes commonly used in the investigation of transcellular transport of ions and water and much work has been done to characterize these processes (*see* reviews by Leaf, 1965, and Sharp & Leaf, 1966). However it has proved difficult to correlate transepithelial movements of ions and water with changes in the composition of the epithelial cells believed responsible for this transport, largely because of the morphology of the tissue. The bladder consists of three distinct layers: the epithelial cells themselves, a supporting connective tissue matrix containing bundles of smooth muscle cells and nutrient blood vessels, and an overlying layer of serosal cells. Though

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attempts have been made to measure intracellular composition under a variety of conditions by analyzing the whole bladder (e.g. Frazier, Dempsey & Leaf, 1962; Essig & Leaf, 1963; Finn, Handler & Orloff, 1966; Herrera, 1968), interpretation of such experiments has proved difficult.

Recently, Gatzy and Berndt (1968) described a technique by which epithelial cells could be scraped from the urinary surface of bladders previously incubated in sodium Ringer's collagenase. Such scraped cells could then be suspended in medium and incubated under a variety of conditions.

The present paper presents results from experiments in which isolated epithelial cells were obtained using a slightly modified technique to that described by Gatzy and Berndt (1968). These support the claim that such cells remain viable during subsequent incubation. A major disadvantage of this technique, however, is that cells so isolated must be bathed on both their mucosal and serosal surfaces with the same solution. Since much important information characterizing transepithelial transport has been obtained by exposing the mucosal and serosal surfaces of bladders to solutions of differing compositions, a simple method has been developed for scraping cells from bladders after they have been mounted in chambers. This technique has the further advantage that responses to hormones and drugs can be monitored on the same epithelial cells subsequently analyzed. Evidence is provided that under these experimental conditions the calculated intracellular values for the epithelial cells truly reflect their composition.

Materials and Methods

Solutions

Solutions were prepared from analytical grade reagents. Sodium Ringer's contained (in mM) Na^+, 117; K^+, 3.5; Ca^{++}, 1.0; Cl^-, 117; glucose, 10; buffered at pH 7.8 by HPO_4^{2-}, 2 mM. Calcium-free sodium Ringer's was prepared by omitting $CaCl_2$, 1 mM. Choline-chloride Ringer's contained (in mM) choline, 112; K^+, 7.5; Ca^{++}, 1.0; Cl^-, 117; glucose, 10; buffered at pH 7.8 by HPO_2^{2-}, 2 mM.

Inulin-carboxyl-^{14}C and inulin-methoxy-^{3}H were obtained from New England Nuclear Corporation, ouabain from Calbiochem, and crude collagenase from Worthington Biochemical Corporation. When crude collagenase was used it was dissolved just before use in calcium-free sodium Ringer's to give a concentration of 400 mg/liter. Amiloride was the gift of Merck, Sharp and Dohme.

Procedure

Bladders from toads of the species *Bufo marinus*, obtained from the Dominican Republic (National Reagents, Inc., Bridgeport, Conn.) were used in all experiments. Toads were doubly pithed and their hearts immediately perfused with sodium Ringer's.

As the liver distended it was cut to allow drainage of blood from the animal. After several minutes, vessels in the hemibladders were free of blood and the hemibladders were then removed and placed in sodium Ringer's.

(I) Experiments with Sheets of Isolated Epithelial Cells

(a) Determination of Cellular Composition. Hemibladders were placed in a beaker containing calcium-free sodium Ringer's with collagenase (400 mg/liter) and incubated in a water bath at 25 °C. Oxygen bubbling through the medium provided adequate stirring of the solution. After 40 min, a small volume of a concentrated solution of $CaCl_2$ was added to the medium to provide a final calcium concentration of 1 mM.Twenty min later, bladders were removed from the Ringer's with collagenase and transferred to fresh oxygenated sodium Ringer's where they remained until scraped.

The epithelial cells were scraped from the bladders using a glass microscope slide. After all the bladders in any one experiment had been scraped, a process which might take up to 20 min, the scrapings were repeatedly aspirated in a Pasteur pipette to obtain uniform mixing of the tissue. They were then transferred to a centrifuge tube and washed by centrifugation for 10 min in sodium Ringer's at 3,000 rpm in a clinical centrifuge. The supernatant and any debris settled at the bottom of the tube were removed, the cells resuspended in fresh sodium Ringer's and the centrifugation repeated.

After the supernatant had been discarded, aliquots of cells were transferred to 5-ml portions of sodium Ringer's containing 1 μC inulin-carboxyl-^{14}C, contained in covered beakers in a water bath at 25 °C. Oxygen was bubbled through the solutions, in which the cells remained for up to 120 min. At the end of incubation, medium and cells were transferred to lucite centrifuge tubes similar in design to those described by Burg and Orloff (1962) and the tubes centrifuged in a Sorval ultracentrifuge for 20 min at 31,000 × g. The temperature of the centrifuge was held at 17 °C giving a temperature in the medium of 25 ± 2 °C. During centrifugation the cells form a pellet in the narrow cylinder at the bottom of the tube. So long as the cells remain suspended in the medium, they are freely bathed with the sodium Ringer's through which oxygen had been bubbled prior to centrifugation. Once they have entered the narrow cylinder along with a small portion of medium trapped with them, it is possible that they may become anoxic. However, any redistribution of ions or water between the cells and the trapped medium which might occur at this time, would not affect the results since the inulin content of the column would not be altered.

After centrifugation the supernatant was removed and the packed column of cells extruded into weighed tubes for analysis.

(b) Measurement of Cellular Oxygen Consumption. The oxygen consumption of the isolated sheets of epithelial cells was measured by a method similar to that described by Gatzy and Berndt (1968). Pooled epithelial cell scrapings were added to a small volume (1.25 ml) of sodium Ringer's in manometer flasks which were mounted on all-glass Gilson differential respirometers. A second flask, containing the same volume of sodium Ringer's but no tissue, served as the control for each respirometer. The center wells were filled with 0.2 ml of 20% KOH and contained a small roll of filter paper to increase the area available for CO_2 absorption. The all-glass differential respirometers allow the use of oxygen as the gas phase and provide more accurate correction of the manometer readings for any fluctuations in temperature than does the conventional Warburg apparatus.

Oxygen consumptions were measured over a 2-hr period, the flasks were then dismantled and tissue and medium aspirated into weighed tubes. The flasks were then rinsed several times with distilled water, the rinsings being transferred to the same weighed tube. Control flasks without tissue, were treated similarly. All tubes were dried

to constant weight at 105 °C in a hot-air oven, allowed to cool to room temperature, and reweighed. The dry weights of tissue were obtained by correcting the increase in weight of the tubes containing tissue and medium for the weight of the evaporated medium in the control tubes, and the oxygen consumptions of the tissue were expressed in μliters per hr per mg tissue dry weight.

(II) Experiments in Which Cells Were Scraped from Mounted Bladders

For these experiments the two hemibladders from the same toad were mounted in identical chambers, each of which provided an area of 8.04 cm^2 across which transport could occur. In order to obtain sufficient tissue for subsequent analysis, large toads (usually weighing 350 gm or more) were used and the hemibladders mounted loosely with the serosal surface supported on nylon mesh. The mucosal surface was bathed with 20 ml of solution, the serosal with 19 ml, the difference in hydrostatic pressure ensuring that the hemibladders lay firmly against the nylon mesh. The solutions were aerated throughout the experiments unless otherwise indicated. The usual procedure was to bathe both surfaces of each hemibladder with sodium Ringer's and initially record the open-circuit potential difference. Bladders were then continuously short-circuited by conventional methods and pulses of constant voltage applied to provide a measure of the tissue resistance. These pulses were discontinued during the final 60 min of the experiments to avoid any possibility that they might affect cellular composition. Once short-circuit current had become stable (usually after about 20 min), the chambers were drained, the serosal chamber always being drained first so that the hydrostatic pressure from the mucosal medium would keep the bladder opposed to the nylon mesh, and then refilled with the appropriate solutions, the mucosal chamber being filled before the serosal. The final solutions, which were present for at least 60 min before the chambers were dismantled, contained labeled inulin. In experiments in which bladders were bathed on both surfaces with identical solutions, inulin-carboxyl-^{14}C was present in all solutions. When the composition of the mucosal and serosal solutions differed, inulin carboxyl-^{14}C was present in the mucosal solutions and inulin-methoxy-^{3}H in the serosal solutions. The measurements indicated that these isotopes do not cross the bladder. It was, therefore, possible to calculate the relative contributions of each solution to the extracellular contamination of the epithelial cells.

When the chambers were dismantled, the portion of the hemibladder which had been exposed in the chamber to the solutions, together with its supporting nylon mesh, was cut from the chamber and carefully blotted on Whatman #542 filter paper. The mesh was removed, the bladder blotted several more times until no visible moisture was transferred to the filter paper and then placed, mucosal surface up, on a Pyrex petri dish. A glass slide was used to scrape the epithelial cells from the bladder, and the scrapings were then transferred with a Pasteur pipette to tared tubes for subsequent analysis.

Tissue Analysis

The tissue water content was determined by drying to a constant weight in a hot air oven at 105 °C and equating the loss of weight during this procedure with the water content of the tissue. It was found that, as with other tissues (Little, 1964), tissue weight remained constant after 2 hr.

The dried tissue was then extracted overnight (16 hr or more) in 10 ml of 0.1 M nitric acid. This provided ample time for the extraction of the ions (Little, 1964). Sodium and potassium were measured in the acid extracts with an EEL flame photometer using

external standards prepared in 0.1 M nitric acid. There was no evidence of interference between the ions in the concentrations encountered. Chloride was measured in acid extracts using a Cotlove titrator (Cotlove, Trantham & Bowman, 1958).

Inulin was measured by liquid scintillation counting. A 2-ml portion of the 0.1 M HNO_3 tissue extract was neutralized with 0.2 ml of 1 M NaOH. When only inulin-carboxyl-^{14}C was present, 15 ml of dioxane fluor was added and the samples counted in a Nuclear Chicago Scintillation counter. Samples (0.1 ml) of medium were diluted by the addition of 3 ml of 0.1 M HNO_3, and 2 ml of this was then neutralized and counted in the same way as the tissue samples. When both inulin-carboxyl-^{14}C and inulin-methoxy-^{3}H were present, the procedure was similar except that 15 ml of Bray's solution (Bray, 1960) was used and the samples counted in a three-channel Packard Tri-Carb liquid scintillation counter. In all experiments, quenching corrections were made by the channels ratio method.

Expression of Results

All values presented in the tables show the mean ±SEM. For experiments with isolated sheets of epithelial cells, the significances of differences were obtained by performing Student's *t*-test on unpaired data. In experiments in which cells were scraped from hemibladders which had been mounted in chambers, differences between groups were evaluated using analysis of paired data. The tissue values were corrected for contamination with extracellular fluid using the assumption that isotopically labeled inulin equilibrated in the extracellular space and that the ions in this space were at the same concentrations as in the bulk of the medium. Intracellular water content is expressed as kg H_2O per kg dry weight. The ion contents of the cells are shown in mEquiv per kg dry weight and their concentrations in mEquiv per kg intracellular H_2O.

Microscopy

Phase and electron microscopy were utilized to check the purity of fractions and to assess the structural integrity of cells throughout the isolation procedure. Samples were fixed initially by addition of glutaraldehyde to a final concentration of 1% in the suspending medium. This was followed by conventional treatment in a 1% glutaraldehyde solution in Sorenson's phosphate buffer, post-fixation in 1% OsO_4, dehydration in a graded ethanol series, and embedment in an Epon-Araldite mixture (DiBona, Civan & Leaf, 1969). Sections were cut on a Reichert OmU2 ultramicrotome at thicknesses of 1 to 2 μ for phase microscopy and 250 to 500 Å for electron microscopy. Sections for electron microscopy were stained with uranyl acetate and lead citrate and examined in a Philips EM-200 electron microscope.

Results

Isolated Sheets of Epithelial Cells

(I) Morphology

Epithelial cell fractions from the collagenase procedure were examined by both phase and electron microscopy (Figs. 1a, 1b and 2). Only occasionally were red blood cells, fibroblasts or smooth muscle cells found; accurate

estimation of the contamination is scarcely possible but, on a volume basis, 2 to 3% represents a certain upper limit of the submucosal component in the epithelial cell fraction. Comparison of isolated epithelial sheets with epithelia of numerous intact preparations from this laboratory indicated that little or no adverse effect on cell architecture had been imposed by the isolation procedure. Samples were taken throughout the course of the study for microscopic examination; the results cited above and illustrated in Figs. 1a, 1b and 2 were found most consistently.

(II) Cellular Composition and Oxygen Consumption

Table 1 shows the results of experiments in which isolated sheets of epithelial cells were incubated for up to 2 hr at 25 °C in oxygenated sodium Ringer's. From 30 to 120 min, the cells were clearly in a steady state with respect to water and ions. Similarly, over a 2-hr period, tissue oxygen consumption remained relatively constant (Table 2).

Table 1. *Cellular composition as a function of incubation time*

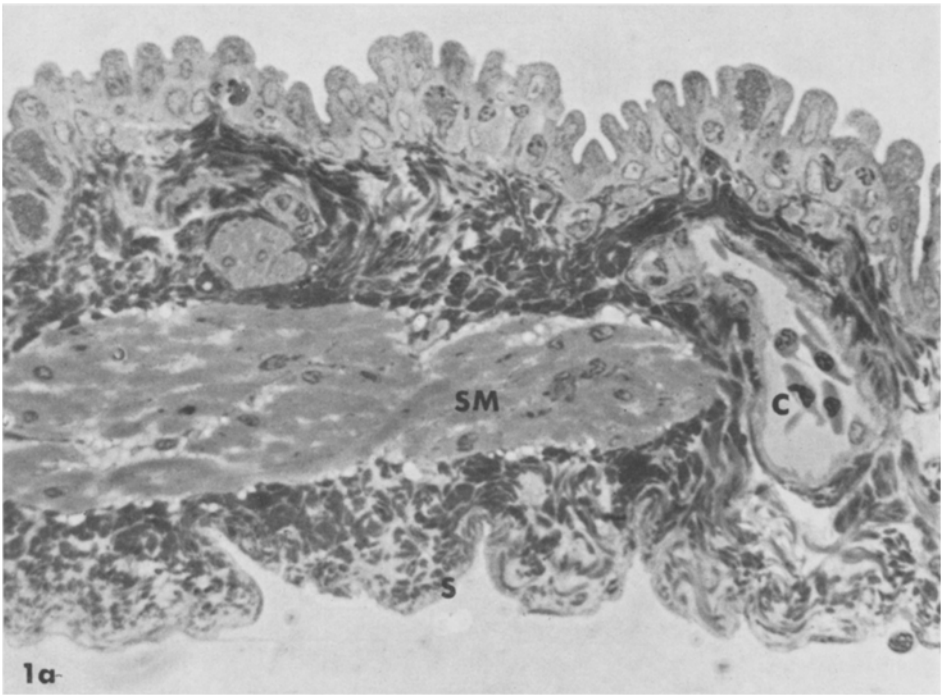
Time of incubation (min)	Extracellular space (percentage of tissue H_2O)	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg intracellular H_2O)		
			Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
0	38.3±2.3	3.91±0.05	243±16	429±15	246±5	62±5	109±3	63±1
30	44.7±4.0	3.31±0.06	208±18	457±16	233±12	63±6	138±3	70±4
60	43.3±3.4	3.17±0.11	203±14	431±18	230±13	65±6	137±8	73±6
120	43.7±2.4	3.23±0.09	199±10	448±7	237±9	62±4	139±4	73±3

Eight observations in each group from pooled cells scraped from 96 hemibladders of 48 toads. Four separate experiments including two observations at each time were performed.

Table 2. *The oxygen consumption of isolated sheets of epithelial cells incubated in sodium Ringer's*

	O_2 Consumption (μliter/hr/mg tissue dry wt)
After 60 min	4.71±0.37
After 120 min	4.29±0.13
p	>0.10

Eight observations in each group from pooled tissue scaped from 48 hemibladders from 24 toads. Two separate experiments with four observations at each time were performed.



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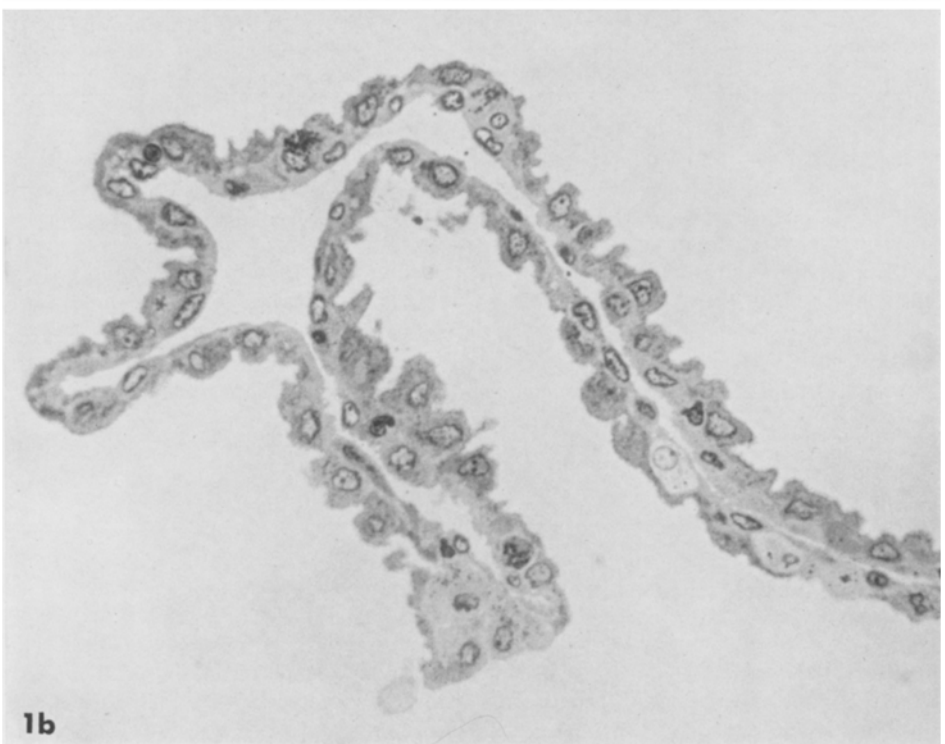

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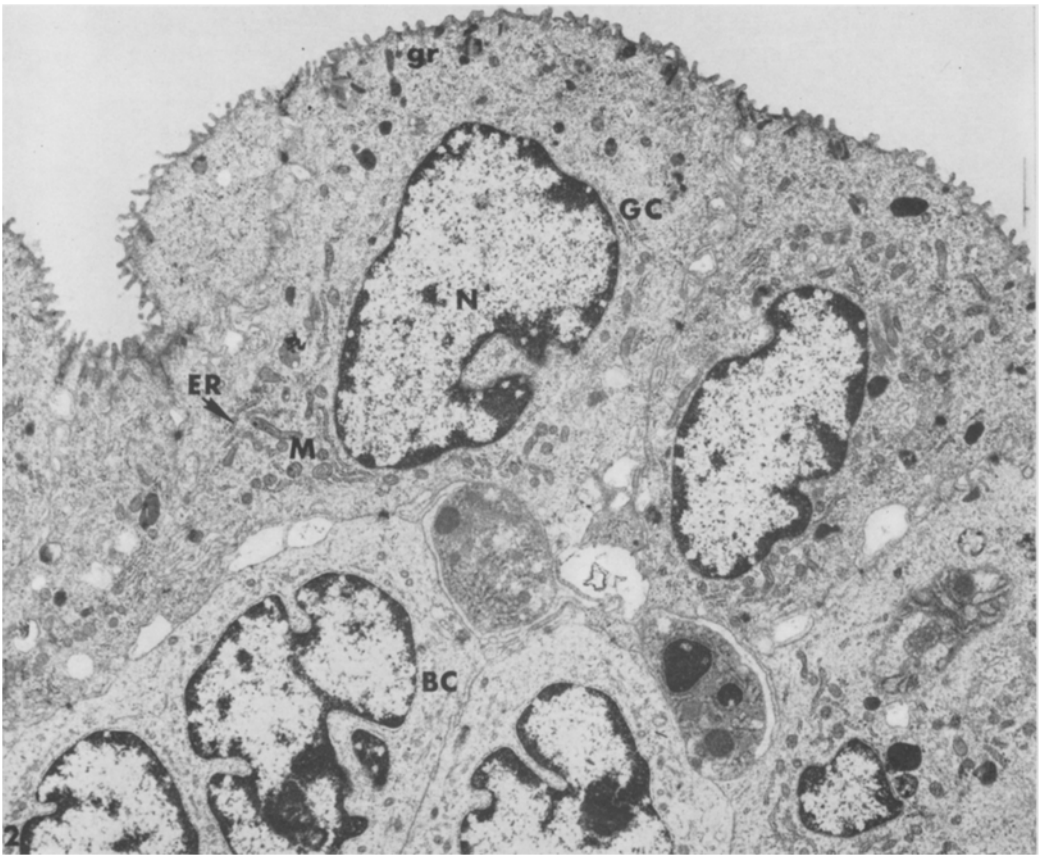


Fig. 2. Electron micrograph of toad bladder epithelial cells after collagenase treatment and removal from submucosa. The granular cells *GC* shown have undergone no apparent structural changes; nuclei *N*, mitochondria *M*, granules *gr*, and saccules of the rough endoplasmic reticulum *ER* all appear as they do in similarly fixed untreated preparations. There is no dispersal of the cytoplasmic matrix or vacuolation as is commonly seen when cells are isolated with less gentle procedures (e.g., calcium depletion). The basal cells *BC* also look as they do in intact bladders where they also show slightly less cytoplasmic density than the granular cells. × 7,000

Fig. 1. (a) Phase micrograph of toad bladder illustrating the morphological characteristics of this preparation in the absence of experimental manipulation. The mucosal epithelium (top) comprises, in this view, about one-fifth of the total preparation thickness; this relationship is highly variable however. Submucosal elements illustrated include bundles of smooth muscle *SM*, a capillary *C* with contained blood cells, collagen fibers (the dense component of the matrix) and the contramucosal lining – a thin serosa *S*. × 600. (b) Phase micrograph of epithelial scraping after collagenase treatment. The strip of mucosal cells shown typifies the nature of these preparations – irregular sheets containing 50 to several hundred cells attached to each other as they are in the intact bladder. There is no evidence of swelling or vacuolation. × 600

Table 3. *Changes in composition of isolated epithelial cells during preparation*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg intracellular H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Without incubation	3.95 ± 0.06	237 ± 7	$334 > 11$	252 ± 2	60 ± 2	85 ± 2	64 ± 1
With incubation	2.63 ± 0.09	147 ± 8	329 ± 11	172 ± 9	56 ± 3	125 ± 4	67 ± 2
p	<0.001	<0.001	>0.70	<0.001	>0.20	<0.001	>0.20

One experiment with six observations in each group from pooled cells scraped from 36 hemibladders of 18 toads.

It will be seen that the initial samples taken at the end of the preparation of the isolated sheets of cells before they were incubated in oxygenated sodium Ringer's inulin-^{14}C, appeared to be somewhat swollen (time zero in Table 1). These cells had simply been placed in sodium Ringer's with inulin-^{14}C and immediately centrifuged at $31{,}000 \times g$ for 20 min. It was possible that the cells were not swollen but appeared so because inulin had failed to equilibrate in the extracellular space under these conditions. Alternatively the cells might actually have swollen during their preparation. About 40 min elapsed from the time the first cells were scraped from the bladders to the time cells were ready for incubation in oxygenated medium. During this period the cells were suspended in sodium Ringer's but not aerated or oxygenated, and they spent two successive 10-min periods packed in a centrifuge tube at 3,000 rpm.

We performed an experiment to resolve these possibilities; the results are shown in Table 3. After initial incubation in oxygenated sodium Ringer's with collagenase for 60 min, hemibladders were transferred to oxygenated sodium Ringer's with inulin-^{14}C where they remained until scraped. During subsequent scraping, centrifugation and washing, the tissue was constantly exposed to inulin in sodium Ringer's. The isolated sheets of epithelial cells, therefore, spent 30 to 40 min with inulin-^{14}C and were fully equilibrated with the extracellular marker. A portion of the cell scrapings were transferred to the lucite centrifuge tubes and centrifuged at $31{,}000 \times g$ for 20 min in the usual way, before analysis. The remaining scraped cells were incubated at 25 °C for 60 min in oxygenated sodium Ringer's with inulin-^{14}C before centrifugation and analysis, as in the 30 to 120 min periods shown in Table 1.

Table 4. *Effects of collagenase on intracellular composition*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg intracellular H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Control	3.09 ± 0.20	191 ± 18	460 ± 11	223 ± 13	62 ± 6	151 ± 6	73 ± 1
Collagenase-treated	2.91 ± 0.05	178 ± 12	431 ± 9	220 ± 13	61 ± 4	148 ± 4	76 ± 4
p	>0.40	>0.50	>0.05	>0.80	>0.80	>0.60	>0.40

One experiment with six observations in each group from cells scraped from 12 hemibladders of 12 toads.

It is clearly shown in Table 3 that, in spite of adequate time for inulin-^{14}C to equilibrate in the tissue, the water, sodium and chloride contents of the cells analyzed before incubation are significantly greater than those of tissue incubated for 60 min in oxygenated medium before analysis.

It will be noted that the water and electrolyte values given in Table 3 for tissue incubated in oxygenated medium for 60 min are somewhat different from those shown in Table 1. It has been noticed that there are appreciable differences in electrolytes and water between batches of toads obtained in the same season, as well as seasonal variations. It is therefore essential in these studies to provide adequate controls with every experiment and to make comparisons only between results obtained with the same pooled tissue.

The effects of collagenase itself on the behavior of toad bladders were examined by mounting hemibladders in chambers and exposing a quarter-bladder on both mucosal and serosal surfaces to the same concentration of collagenase as was used to isolate epithelial sheets. The other quarter-bladder bathed with sodium Ringer's without collagenase served as a control. Tissue exposed to collagenase maintained a normal short-circuit current (SCC), and the SCC after vasopressin (100 mU/ml) increased to the same extent as in the control hemibladder. Similarly, amiloride (10^{-5} M) produced the same prompt fall in SCC when added to the mucosal medium bathing both quarterbladders. It was, therefore, concluded that collagenase treatment did not prevent the bladders from transporting sodium in the usual way.

To determine if the presence of collagenase altered the composition of epithelial cells, an experiment, the results of which are shown in Table 4,

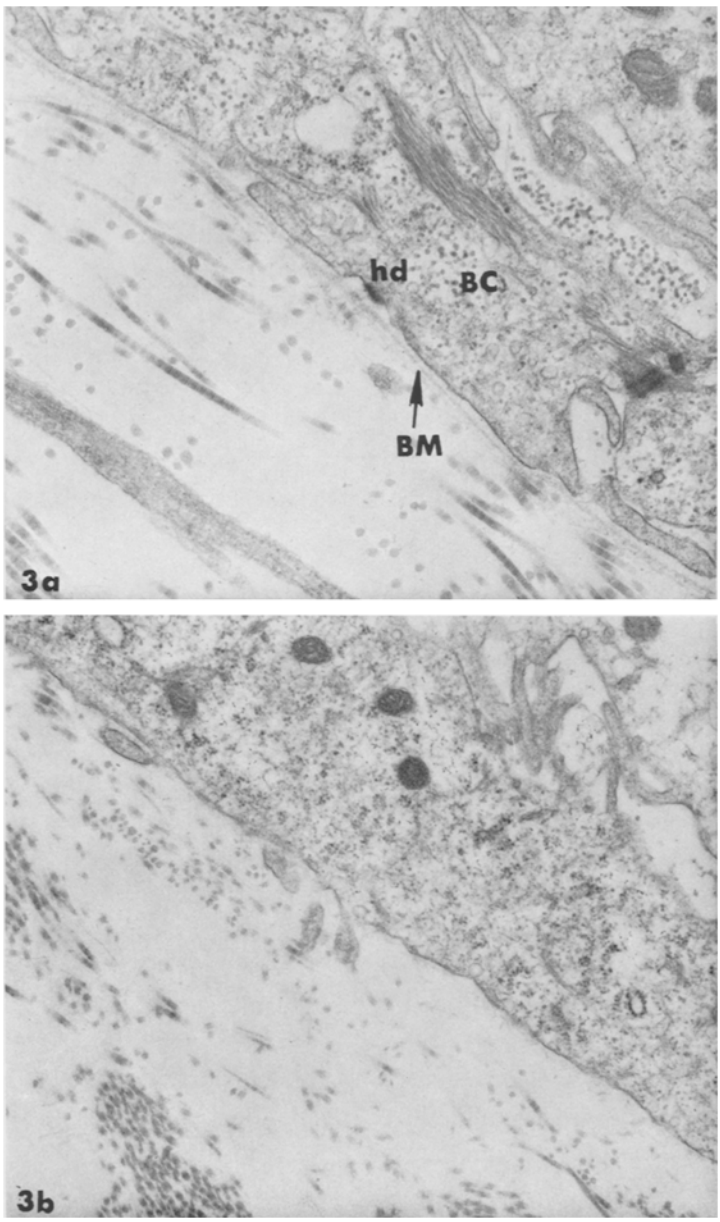


Fig. 3. (a) Electron micrograph of the epithelial-submucosal interface to illustrate the 150 Å thick basement membrane *BM* present in the absence of collagenase treatment. The plasma membrane of the basal cell *BC* is in close apposition. Occasional hemidesmosomes *hd*, focal attachment sites between cells and basement membrane, are also observed. × 17,000. (b) Electron micrograph of an interfacial zone from collagenase treated preparation. Both Figs. 3a (control) and 3b (collagenase treatment) are taken from paired quarterbladders of a single experiment. Note that the basement membrane is no longer visible here. While it is necessary to use fairly high magnification to demonstrate the presence or absence of basement membrane in this tissue, examination of numerous regions has established that at least 95% of this otherwise complete structure is removed by the described procedure. × 17,000

Table 5. *The effects of ouabain on composition of isolated epithelial cells*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg intracellular H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Control	3.51 ± 0.09	165 ± 8	420 ± 10	235 ± 7	48 ± 3	120 ± 4	67 ± 1
Ouabain (10 mM)	3.37 ± 0.06	395 ± 5	176 ± 8	251 ± 5	118 ± 3	49 ± 3	74 ± 1
p	>0.10	<0.001	<0.001	>0.05	<0.001	<0.001	<0.001

Twelve observations in each group from pooled tissue scraped from 72 hemibladders of 36 toads. Three separate experiments with four observations in each group were performed.

was performed. It is clear that previous exposure to collagenase did not affect tissue composition. It seemed reasonable to conclude, therefore, that exposure to collagenase in the concentrations used here, did not affect the properties of the cellular membranes responsible for the maintenance of the normal cellular volume and ionic environment. This conclusion is consistent with the appearance of the cells themselves, presented earlier. Electron microscopy revealed that exposure to collagenase resulted in the digestion of the basement membrane material, the overall appearance of the bladder components being otherwise unchanged and the highly organized fibrillar collagen of the matrix not noticeably affected. This is illustrated in Fig. 3a and b. These findings suggest that the epithelial cells removed from the bladder after collagenase treatment should be relatively little damaged during their separation from the underlying connective tissue, and this may explain the viability of the preparation during subsequent incubation.

Finally, to demonstrate the fact that changes in intracellular composition can indeed be detected in isolated sheets of epithelial cells, Table 5 presents results obtained when isolated sheets were incubated in the presence of 10 mM ouabain. The epithelial cells of the toad are relatively insensitive to cardiac glycosides and this high concentration of ouabain was selected to obtain a maximal effect. Such a concentration virtually abolishes SCC in mounted hemibladders. Clearly the presence of ouabain produced a large increase in cellular sodium ($+230$ mEquiv/kg dry weight) and a similar decrease in cellular potassium (-244 mEquiv/kg dry weight). Potassium

measured in these experiments was clearly intracellular (since the medium concentration is so low that extracellular contamination with potassium is negligible). The equivalence and reciprocal nature of the changes in sodium and potassium lends strong support to the claim that change in content of these cations occurred in the same compartment of the tissue, namely the non-inulin intracellular space.

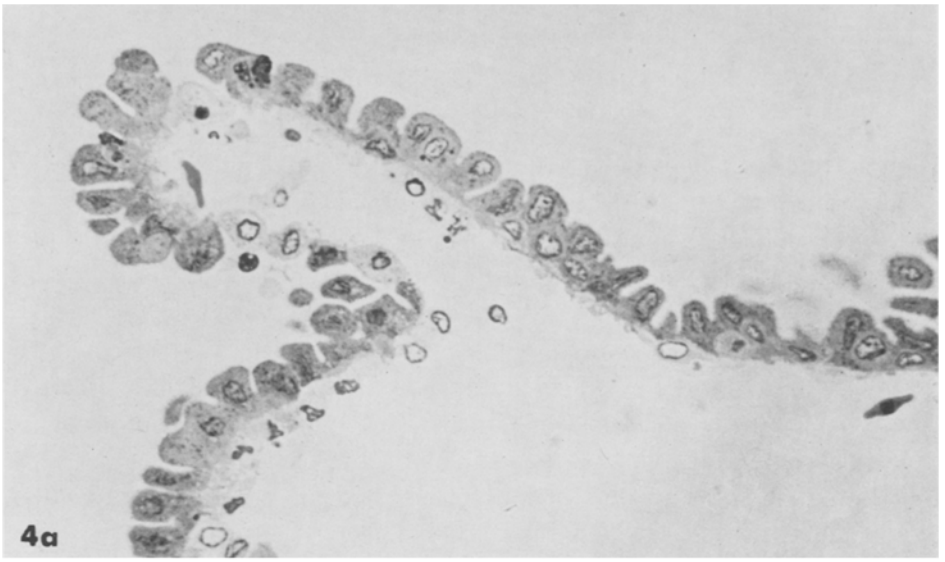
Although ouabain produced striking changes in cellular sodium and potassium, the cellular water and chloride contents were not appreciably altered. These findings are similar to results obtained in slices of mammalian renal cortex (Kleinzeller & Knotkova, 1964; Willis, 1966; Macknight, 1968; Whittembury, 1968) in which cells appear to regulate their volume by a mechanism insensitive to cardiac glycosides.

Epithelial Cells Scraped from Hemibladders Following Their Incubation in Chambers

(I) Morphology

Phase-microscopic examination of epithelial scrapings and the remaining submucosal strips (Fig. 4a–c) indicated that at least 95% of the cells were removed when the bladdere were scraped after being mounted in chambers and without pre-treatment in collagenase. Electron microscopy of these cells (not shown) again showed preservation of structural integrity but with retention of the basement membrane on the basal surface of the epithelium in most cases. Although the preparative procedures for microscopy tend to enhance the presence of contaminating elements (since centrifugation is necessary for the first few changes of solutions), very few submucosal elements were detected in the final epithelial cell fractions.

Fig. 4. (a) Phase micrograph of epithelial scraping from chamber experiment (no collagenase treatment). As in the previous illustrations, the epithelial cells are removed in fairly large sheets with no apparent damage. There is, perhaps, a slightly higher frequency of broken basal cells in these samples but, in either case, this seems to be less than 10% of that cell population. Virtually no damage to granular, mitochondria-rich or goblet cells, the only types which reach the mucosal surface, is observed. ×600. (b) Phase micrograph of submucosa after scraping as in Fig. 4a. The mucosal surface (top) is well-cleaned of epithelium and there is no evidence of disruption or loss of submucosal elements. ×600. (c) Phase micrograph of submucosa as in Fig. 4b. This illustration provides an example of the rare instances where mucosal cells (between arrows) were left behind by the scraping procedure. Examination of approximately 1-1/2 linear cm of the scraped surface suggests that less than 5% of the mucosal epithelium was not removed in these experiments. ×600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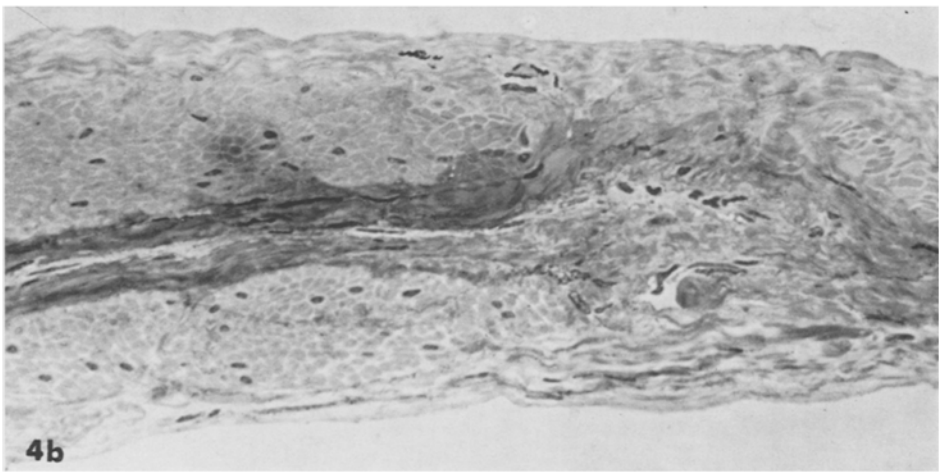
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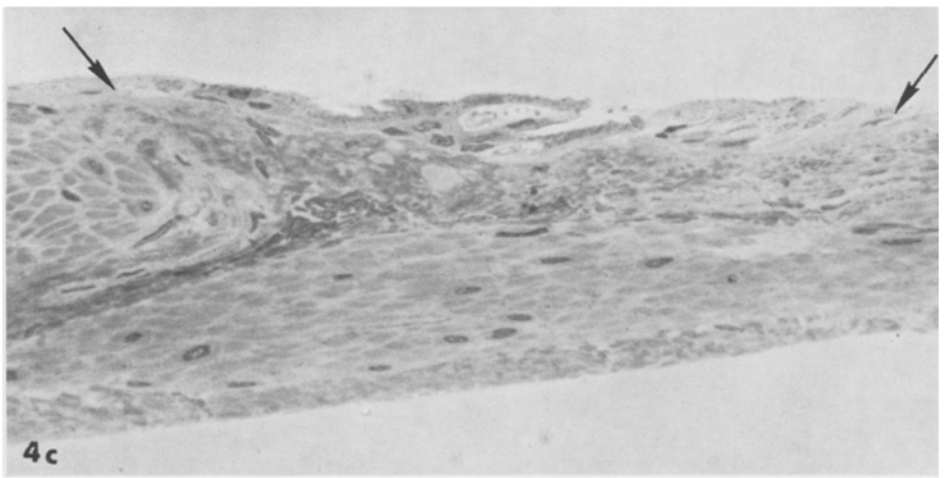
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(II) Cellular Composition

The two hemibladders from the same toad were used in each individual experiment, one hemibladder acting as the appropriate control for the other. That hemibladders from the same toad are indeed well matched in respect to their intracellular composition is shown in Table 6. In these experiments, both hemibladders were bathed on their mucosal and serosal surfaces with sodium Ringer's. There were no significant differences in water or electrolyte composition when paired analyses were performed. Furthermore, the potassium contents of the epithelial cells were similar to those of the isolated sheets of epithelial cells shown earlier. This indicates that intracellular fluid was not being squeezed out of submucosal cells (e.g., muscle cells) during the scraping. If this had occurred, the potassium content of the epithelial cells would have appeared to be higher.

A potential problem in interpretation of results obtained when epithelial cells are scraped from bladders in this way is that the extracellular marker used may not penetrate into the total extracellular space and therefore results obtained may overestimate the intracellular water, sodium and chloride. Such a criticism might be especially relevant in experiments where transepithelial transport was occurring, for sodium together with chloride might accumulate in an extracellular compartment relatively inaccessible to inulin. This problem was touched on in the preceding section when results with isolated sheets of epithelial cells were discussed and it was concluded that the effects of ouabain in this preparation made such a possibility unlikely. However, the isolated sheets of epithelial cells might offer fewer barriers to diffusion of inulin than whole bladders. It is felt,

Table 6. *A comparison of the composition of epithelial cells scraped from paired hemibladders mounted in chambers for 2 to 3 hr and bathed on both surfaces by sodium Ringer's*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg intracellular H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
	3.13 ± 0.12	162 ± 7	458 ± 10	209 ± 13	52.1 ± 2.7	147.5 ± 4.5	66.9 ± 3.1
	3.30 ± 0.15	155 ± 13	462 ± 13	214 ± 14	46.9 ± 3.1	141.4 ± 5.2	64.6 ± 2.0
Difference	0.17 ± 0.09	7 ± 14	4 ± 5	5 ± 11	5.3 ± 3.5	6.1 ± 4.1	2.3 ± 3.7
p	>0.10	>0.60	>0.40	>0.60	>0.10	>0.10	>0.50

Eight experiments with eight observations in each group from eight hemibladders from eight toads.

however, that limited penetration of inulin into the extracellular space is not a problem in these experiments, for several reasons.

It has been shown that horseradish peroxidase (mol. wt. 40,000) enters the lateral intercellular spaces in toad bladders (Masur, Holtzman & Eggena, 1969), the compartment into which it is probable that sodium entering the cells from the mucosal fluid is pumped (Diamond & Tormey, 1966).Inulin has a molecular weight of 6,000 and one would not therefore predict that it would be excluded from these spaces. Furthermore, since the distribution of inulin in toad bladders reaches a steady state after 20 min (Finn, Handler & Orloff, 1966), it must at this time have become distributed throughout all the accessible extracellular water including that within the lateral intercellular spaces.

We performed experiments to examine this problem directly in cells scraped from mounted bladders; the results are shown in Table 7. Hemibladders were mounted in chambers and bathed on both surfaces by aerated sodium Ringer's containing inulin-^{14}C. Once the SCC had become stable, nitrogen was bubbled through the solutions for 60 min. Under these anaerobic conditions, SCC fell by $80 \pm 3\%$. After 60 min, one hemibladder was removed and the epithelial cells scraped off for analysis. The solutions bathing the other hemibladder were then aerated once again, and SCC rose appreciably in all experiments, the increase after 30 min incubation being $261 \pm 94\%$ of the SCC after anaerobiosis. At this time, the second hemibladder was taken for analysis.

Table 7. *The composition of epithelial cells scraped from paired hemibladders mounted in chambers after anaerobic incubation for 60 min or after anaerobic incubation for 60 min followed by aerobic incubation for 30 min*

	Intracellular water (kg/kg dry wt)	Intracellular ion contents (mEquiv/kg dry wt)			Intracellular ion concentrations (mEquiv/kg intracellular H_2O)		
		Na^+	K^+	Cl^-	Na^+	K^+	Cl^-
Anaerobic	3.67 ± 0.20	222 ± 18	426 ± 13	242 ± 22	60 ± 3	118 ± 5	66 ± 3
Anaerobic and aerobic	3.02 ± 0.22	187 ± 20	403 ± 15	202 ± 21	63 ± 6	138 ± 7	67 ± 3
Difference	0.65 ± 0.24	35 ± 14	23 ± 17	40 ± 13	3 ± 5	20 ± 6	1 ± 3
p	<0.025	<0.05	>0.20	<0.02	>0.50	<0.01	>0.60

Ten experiments with 10 observations in each group from 10 hemibladders from 10 toads.

Preliminary experiments had shown that, as with many other tissues, anaerobic incubation of toad bladder epithelial cells caused tissue swelling with uptake of sodium and chloride, and that this process could be reversed by subsequent oxygenation. This can be seen in Table 7 where epithelial cells from hemibladders exposed to air following anaerobic incubation contained significantly less water, sodium and chloride than cells from hemibladders analyzed after anaerobic incubation. Unlike many mammalian tissues incubated anaerobically, the toad bladder epithelial cells did not lose potassium, though the increase in cellular water content led to a fall in potassium concentration. This observation suggests that anaerobic metabolism can provide sufficient energy for potassium accummulation in this tissue.

If it is assumed that the increase in SCC after oxidative metabolism was restored was caused entirely by movement of sodium from the mucosal to the serosal fluid, the usual cause of the SCC in this tissue (Leaf, 1965), then it is possible to calculate the total increase in sodium movement across the tissue over this 30-min period. To allow direct comparison with the changes in intracellular sodium during the same period, the results have been expressed in mEquiv sodium moved per kg tissue dry weight. It can be calculated that 263 ± 112 mEquiv sodium moved across the bladder at a time when the intracellular content of sodium dropped by a mean value of 35 ± 14 mEquiv per kg dry weight. It, therefore, proved possible to detect a significant decrease in intracellular sodium content at a time when transepithelial transport of sodium had increased appreciably, a result which argues strongly against the possibility that sodium transported from mucosa to serosa is trapped in an inulin-inaccessible extracellular space, and thereby leading to overestimation of the intracellular sodium, chloride or water.

Discussion

The results of the experiments presented in this paper provide convincing evidence for the validity of analysis of epithelial composition in cells scraped from toad bladders whether these cells are obtained from bladders treated with collagenase and subsequently incubated, or whether they are scraped from bladders which have been mounted in chambers, and then analyzed directly. Both the morphological studies and tissue analysis support this conclusion. For many purposes the isolated sheets of epithelial cells provide an excellent preparation, and they offer the great advantage that the oxygen consumption of the transporting cells alone can be directly determined under a variety of experimental conditions. Furthermore, one

is not limited, as one is with paired hemibladders, to an analysis of only one experimental situation in any one experiment, for the pooled cells can be distributed to a number of beakers and subjected to a variety of procedures. However, they have two clear disadvantages: (1) it is not possible to monitor directly the response of sodium transport of the isolated epithelial cells to hormones or drugs in the same preparation that will subsequently be analyzed for its intracellular composition; and (2) it is not possible to bathe the two surfaces of the cells with solutions of differing composition. Bladders mounted in chambers suffer from neither of these disadvantages.

One major problem in all experiments of this type in which tissue water and electrolytes are analyzed is that no conclusions are possible about the localization within the cells of the ions and water measured. Clearly, the calculation of an intracellular ion concentration by dividing a cellular ion content by the total cellular water may have little relevance to the real ion concentrations within the cell. In addition, it is not possible in this type of experiment to come to any conclusions about the relative contributions of the cell types making up the epithelial layer to the overall cellular composition derived. The predominant cell type in the toad bladder epithelium is the granular cell, but three other cell types – mitochondria-rich, goblet and basal cells – are present in significant numbers.

In spite of these limitations, it would seem reasonable to suppose that analysis of the intracellular composition of epithelial cells scraped from toad urinary bladders will provide additional insights into the mechanisms involved in ion movements and volume regulation in transporting epithelia. The following paper (Macknight, Leaf & Civan, 1971) provides a demonstration of this.

The technical assistance of Miss Bonnie Lord is gratefully acknowledged. This work was supported in part by grants from the John A. Hartford Foundation, Inc., and by U.S. Public Health Service grants HE-06664 from the National Heart Institute and A.M.-04501 from the National Institute of Arthritis and Metabolic Diseases. Dr. Macknight is a Public Health service International Postdoctoral Research Fellow (TW 01485-01). Dr. Civan is an Established Investigator of the American Heart Association.

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