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LOCALIZATION AND CHARACTERIZATION OF TRANSPORT-RELATED ELEMENTS IN THE PLASMA MEMBRANE OF TURTLE BLADDER EPITHELIAL CELLS

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Summary

A mixed membrane preparation obtained from turtle bladder epithelial cells contains ($Na^+ + K^+$)-ATPase, adenylate cyclase and protein kinase, which interact with ouabain, norepinephrine and cyclic AMP, respectively. When such a preparation is obtained from bladders which had been preexposed to serosal fluids containing the tritiated form of 4,4′-diisothiocyano-2,2′-disulfonic stilbene, the subsequently isolated membrane proteins are enriched in tritium as well as in the afore-mentioned enzymes, none of which is inhibited. Free-flow electrophoresis separates the mixed membrane preparation into two distinguishable groups: one, construed as apical membranes, is enriched in norepinephrine-sensitive adenylate cyclase and cyclic AMP-sensitive protein kinase; the other, construed as basal-lateral membranes, is enriched in ouabain-sensitive ATPase and 4,4′-diisothiocyano-2,2′-disulfonic stilbene-binding proteins.

The physiological counterparts of these enzymatically defined membrane markers are the mucosal sidedness of the transport effects of norepinephrine and cyclic AMP derivatives and the serosal sidedness of the transport effects of ouabain and disulfonic stilbenes in the intact turtle bladder. The discreteness and ion selectivity of each membrane-bound, transport-related element are discussed in relation to the corresponding characteristics of each transport

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Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-disulfonic stilbene; SITS, 4-acetamido-4′-isothiocyanodisulfonic stilbene.

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process in vivo; the possibility of regulation of anion transport by adenylate cyclase-protein kinase system is also discussed.

Introduction

The present study was undertaken to obtain evidence of the apical or basal-lateral membrane location of certain elements, each of which is or can be related to an ion-selective pump or path in one of the plasma membranes of turtle bladder epithelial cells. The presence of discrete ion-selective pumps and paths in each membrane has previously been established physiologically on the basis of a mutual independence of the reabsorptive flows of Na^+, Cl^- and HCO_3^- in short-circuited bladders [1—15] and on the basis of the mucosal or serosal sidedness of action of certain agents (transport effectors) on these ion flows [6—15]. These transport effectors, also used in the present experiments, include ouabain, norepinephrine, cyclic AMP derivatives, and the disulfonic stilbenes (4,4′-diisothiocyano-2,2′-disulfonic stilbene (DIDS), or 4-acetamido-4′-isothiocyanodisulfonic stilbene (SITS)).

It was thought that the ion-selective pumps or paths would be defined biochemically if each transport effector would change the enzymatic or ion-binding activity of a specific protein (such as $(Na^+ + K^+)$-ATPase or adenylate cyclase) in an isolated mixed membrane preparation of this tissue. The pump and path elements would be defined topographically as well, if the enzymes were appropriately separated with the apical and basal-lateral membranes during free-flow electrophoresis [16—18] of the original mixed membrane fraction.

Accordingly, such mixed membrane fractions (isolated from epithelial cells of bladders which had been incubated in the presence and in the absence of tritiated DIDS) were tested for the presence of ouabain-sensitive ATPase, norepinephrine-sensitive adenylate cyclase and cyclic AMP-sensitive protein kinase. Finally, free-flow electrophoresis [16—18] was used to resolve the mixed membrane fraction into its basal-lateral and apical subfractions and to identify each subfraction on the basis of its relative enrichment in the aforementioned enzymatic markers, i.e. enrichment in those enzymes which are sensitive to ouabain, norepinephrine, or cyclic AMP.

It will be shown that two membrane fractions were isolated by free-flow electrophoresis. One, migrating toward the electropositive pole and containing ouabain-sensitive $(Na^+ + K^+)$-ATPase and a DIDS-binding protein, is considered to represent the basal-lateral membrane; the other, lower in electrophoretic mobility and containing norepinephrine-sensitive adenylate cyclase and cyclic AMP-sensitive protein kinase activity, is therefore considered to represent the apical membrane of turtle bladder epithelial cells. A possible functional role of these apical membrane enzymes in the physiological regulation of anion transport in vivo will be discussed.

Methods

1. Experiments on the intact bladder

(a) Ion transport. After excision from the turtle, the bladder was mounted in the form of paired hemi-bladder sheets interposed between two identical

Ringer solutions in a double-barreled Rehm-Ussing chamber. The exposed surface area of each hemi-bladder in this chamber was 1.5 cm^2 and the mean dry weight, 15 mg. The monitored transport parameters included the transepithelial potential difference, short-circuiting current (I_{sc}), resistance (R), and in some cases, the unidirectional fluxes of [^{3}H]DIDS. The techniques of surgical excision, mounting, measurement of ion flux and electrical parameters, as well as the impelled circulation and composition of the bathing fluids have been described in detail previously [2]. With this chambered preparation, we were able to test for the reversibility (or irreversibility) of the disulfonic stilbene-induced inhibition of anion transport and for the extent of penetration of the bladder wall by these stilbenes.

In order to determine the effects of preexposure of the intact bladder to ouabain or disulfonic stilbenes on certain characteristics of the subsequently isolated membranes (see items b and c below), the epithelial cell layer was separated from the underlying interstitial layer of the bladder, as described below (see item d).

(b) Pretreatment with SITS or ouabain. Two groups of bladder sacs (eight whole bladders/group) in the normal orientation (serosal surface outside) were filled with and immersed in sodium/Ringer's solutions. The control (untreated) group of bladders was incubated in this system for 4 h. The experimental group was incubated in the same bathing system for 2 h before and 2 h after the addition of SITS to the serosal compartment (final concentration 10^{-4} M). The epithelial cells were then separated from the underlying interstitium (see section d) and each of the epithelial cell suspensions (control and SITS treated) obtained was subjected to the procedures required for isolating the mixed membrane fractions (see section II below).

(c) Pretreatment with [^{3}H]DIDS. For each experiment, twenty bladders were mounted (one at a time) as horizontally oriented flat sheets (each of 8.9 cm^2 of exposed area) between two Lucite hemi-chambers of a single-barreled type of Rehm-Ussing chamber. The lower hemi-chamber was filled with 30 ml of sodium/Ringer's solution containing 0.5% bovine serum albumin (fatty acid free, Sigma) and sealed to provide a closed bathing system for the downward-facing surface of the bladder. The upward-facing serosal (or mucosal) surface of this bladder was covered with 1 ml of sodium/Ringer solution containing 500 μCi of [^{3}H]DIDS at a final concentration of 10^{-5} M for a period of 10 min, during which time the entire chamber was manually swirled every 20 s for proper mixing of the bathing fluids. The DIDS-containing Ringer solution was removed from the upward-facing surface of the bladder and replaced with 30 ml of an albumin-containing (0.5%) Ringer for 10 min. The bladder was removed from the Lucite chamber, immersed in 1 l of the same albumin-rich Ringer solution for 30 min (in order to 'scavenge' any residual [^{3}H]DIDS), and then immersed in 1 l of an albumin-free Ringer for 30 more min to wash out the albumin. Finally the epithelial cells were separated from the interstitium (see section d) prior to isolating the membrane fractions.

(d) Isolation of epithelial cells. Twenty or more whole bladders that had been pretreated with disulfonic stilebenes in the manner described above or twenty control (untreated) bladders were each incubated for 1 h in 500 ml of sodium/Ringer containing 2.0 mM EDTA (in the case of bladder sacs) or in

10—20 ml of EDTA/Ringer (in the case of bladder sheets) to separate the epithelial cells from the interstitium in the manner described by Lipman et al. [19]. Once separated, the epithelial cells of these twenty bladders were collected and pooled. The pooled suspension, maintained at 4°C, was then centrifuged at 10 000 × *g* for 20 min to obtain a pellet of whole cells. The yield of cells was 7—9 g for each set of twenty bladders. These cell pellets were then used as starting material for the membrane isolation.

II. Experiments on membrane fractions of epithelial cells

(a) Isolation of membrane fractions. Starting from the pellet of isolated whole epithelial cells, all subsequent homogenization and fractionation procedures were carried out at 0—4°C. The cell pellet (7—9 g) was suspended in 35—40 ml of a buffered solution (180 mM sucrose, 7 mM triethanolamine-HCl, pH 7.6). Aliquots of this suspension (12—15 ml each) were transferred to a Dounce homogenizer (Braun-Melsungen, Co., Melsungen, F.R.G.) in which the cells were disrupted by 20 strokes of the tight-fitting glass pestle. The resulting cell-free homogenates were combined, diluted with an equal volume of the same sucrose/triethanolamine homogenization solution (to a volume of 50—60 ml), and centrifuged twice at 700 × *g* for 10 min in a Sorvall refrigerated ultracentrifuge (Model RC-2B). The supernatant of the second 700 × *g* spin was then centrifuged for 20 min at 17 000 × *g*. The resulting supernatant (denoted S_1) was kept for subsequent centrifugation; the pellet, resuspended in 5 ml of the sucrose/triethanolamine solution was homogenized by ten strokes of the pestle in the Dounce homogenizer, diluted to 40 ml, and centrifuged at 17 000 × *g* for 20 min to yield a second supernatant (S_2) and pellet. This procedure was repeated to yield a third supernatant (S_3) and pellet. The pellet of these 17 000 × *g* spins consisted of a upper 'fluffy' layer of large membrane fragments and 'light' mitochondria and a lower layer of heavy mitochondria; similar pellets have been found in comparable preparations from kidney cortex [18]. The fluffy layers of large membrane fragments (denoted LM in Table II) were removed and stored at −20°C. The supernatants of the 17 000 × *g* spins (S_1, S_2, and S_3) were combined and centrifuged at 100 000 × *g* for 1 h (in a Beckman Ultracentrifuge Model L5-50) in order to obtain a microsomal pellet, referred to hereafter as the 'mixed membranes'. This pellet was suspended in 10 ml of the solution used for electrophoresis (280 mM sucrose, 8.5 mM triethanolamine, 8.5 mM sodium acetate, pH 7.4) and homogenized with five strokes of the pestle in the Dounce homogenizer. Some aliquots of the resulting suspension of mixed membranes (10—15 mg protein in 2 ml) were assayed for various enzyme activities while others were subjected to the preparative free-flow electrophoresis procedure of Hannig and coworkers [16,17] in the manner recommended by Heidrich et al. [18] for the isolation of kidney membranes.

(b) Electrophoretic separation of membrane fractions. Before injection into the electrophoresis apparatus, the suspension of mixed membranes was centrifuged three times for 10 min at 5000 × *g* in order to remove aggregates. The supernatant of the final centrifugation was homogenized by five strokes of the pestle in the Dounce homogenizer and diluted with 7 ml of the electrophoresis solution (see above) to give a membrane protein concentration of about 2—3 mg/ml. A 5 ml aliquot of this membrane suspension was injected as a fine jet

at a rate of 0.1 ml/min for 50 min into the free-flowing solution between the plates of the electrophoretic chamber of FF-4 (Desaga, Heidelberg, F.R.G.) or the FF-5 apparatus (Garching Instruments, Munich, F.R.G.). During this 50 min period the membrane suspension, moving vertically downward with the free-flowing solution, was exposed to a horizontally oriented electric field of 90 ± 9 V/cm and current of 110 mA at a temperature of 6°C while the effluent fractions (2.5—3 ml each) were collected in 90 test tubes, which were maintained at 0°C throughout the procedure.

III. Analyses

Protein. The protein content in the electrophoretic effluents was estimated in two ways: (i) from the absorbance at 280 nanometers (A_{280}) in a Zeiss PMQ II spectrophotometer and (ii) by the method of Lowry et al. [20] after precipitating the membranes with 10% trichloroacetic acid, washing away the supernatant and dissolving the precipitate with an excess of alkali. Bovine serum albumin solutions were used as standards in the Lowry determinations. Within the range tested, the absorbance (A_{280}) was found to be linearly proportional to the protein content of the membrane-containing samples.

[^{3}H]DIDS binding. For estimating the ^{3}H labelling of membranes in those experiments in which the intact bladder had been preexposed to [^{3}H]DIDS prior to membrane isolation, 100-μl aliquots of fluid were removed from each effluent tube of the electrophoresis apparatus, added to scintillation fluid (Omniflor, 0.4% (w/v); protosol, 10% (v/v), New England Nuclear, Boston, MA), and assayed for radioactivity in a liquid scintillation counter (Beckman Instruments, LS-230).

ATPase. In the assay for the activity of Mg^{2+}-ATPase and (Mg^{2+} + Na^+ + K^+)-ATPase membranes (mixed or electrophoretically separated), described in detail previously [21,22], the incubation fluid consisted of: 3 mM $MgCl_2$, 85 mM NaCl, 15 mM KCl, 50 mM Tris-HCl (pH 7.3); 3.0 mM [γ-^{32}P]ATP (final specific activity 10^6 cpm/μmol ATP); 10^{-4} M ouabain (as indicated); 10^{-4} M SITS (as indicated); and 10—20 μg of membrane protein in a final volume of 100 μl. After an incubation of 10—20 min the reaction was terminated by the addition of 25 μl of 25% (w/v) perchloric acid, the precipitate centrifuged at 15 000 × g for 15 min and the supernatant was analyzed for ^{32}P by a modified Berenblum and Chain technique [23].

Adenylate cyclase. In the assay for the activity of adenylate cyclase in the membrane, according to the method of Bär [24] the incubation fluid consisted of: 50 mM Tris-HCl (pH 7.5 at 37°C); 10 mM $MgCl_2$; 1.0 mg/ml bovine serum albumin; 0.5 mM cyclic AMP; 1 mM EGTA; 1 mg/ml creatine kinase (freshly made); 25 mM creatine phosphate (freshly made); 0.2 mM [α-^{32}P]ATP (final specific activity, 10^7 cpm/μmol); 1 mM GTP; 10 mM NaF (when needed); 10^{-4}—10^{-8} M norepinephrine (when needed); and 10—50 μg of membrane protein in a final volume of 1.0 ml. The reaction was initiated by the addition of [α-^{32}P]ATP to the reaction mixture at 37°C; and stopped after 10 min of incubation by lowering the temperature of the reaction mixture to 0°C and adding 5 μl of a solution containing 20 mM each of cyclic AMP, ATP, and AMP, and 200 mM EDTA (pH 7.0).

The quantity of cyclic [^{32}P]AMP formed was estimated chromatographically

on a thin-layer cellulose sheet impregnated with polyethyleneimine (Polymine, PEI plate) using 0.25 M LiCl as a solvent and determining the ratio of ^{32}P migrating with the cyclic AMP spot to that migrating with the other nucleotide spots. In this solvent, ATP, ADP, and AMP remain close to the origin while cyclic AMP moves rapidly away from the origin. The standard nucleotides used (cyclic AMP, ATP, ADP, and AMP) were located on the plates under ultraviolet light using a Chromatovue, Model cc-20 (Ultraviolet Products Inc.). The adequacy of the ATP generating system (i.e. the creatine phosphate and creatine kinase reagents) was also estimated chromatographically on the PEI plates using 2 N formic acid + 5 M LiCl as the solvent and determining the ratio of ^{32}P migrating with ATP, i.e. [α-^{32}P]ATP to the total ^{32}P (i.e. that migrating with ATP, ADP, AMP, and cyclic AMP). In this solvent system ATP migrates over a small ditance, ADP migrates further, while AMP and cyclic AMP migrate with the solvent front. It was necessary for this ratio to be equal to or greater than 0.85 for the regenerating system to be considered adequate; ratios of 0.90 0.90—0.96 were usually found.

Protein kinase. In the assay for the activity of protein kinase in isolated membranes, by a modification of the method of Kuo [25], the composition of the incubation fluid was the following: 50 mM sodium acetate buffer (pH 6.5); 10 mM $MgCl_2$; 25 μM [γ-^{32}P]ATP (final specific activity, 10^8 cpm/μmol ATP); 25 mM Na_2ATP; 0.3 mM EGTA; 2 mM theophylline; 10—20 mM NaF; $5 \cdot 10^{-8}$—$5 \cdot 10^{-6}$ M cyclic AMP; and 20—50 μg of membrane protein in a final volume of 0.2 ml. The reaction was started by adding the buffered [γ-^{32}P]-ATP-containing mixture (including ATP, magnesium, EGTA, and sodium acetate) to the membrane-containing mixture (including theophylline, NaF or cyclic AMP); and was terminated after an incubation at 30°C for 5 min by the addition of 2.0 ml of 5% trichloroacetic acid (at 0°C). The precipitated membranes were then washed repeatedly on a Millipore filter (HA, 0.45 μm) with a wash solution of 5% trichloroacetic acid, 1.25 mM ATP and 25 mM H_3PO_4. Each filter was then dried, immersed in a 10 ml solution of Instagel, which was then placed in the Beckman, LS-230 Scintillation Counter for 10 min in order to assay the quantity of ^{32}P counts.

Other. In order to ascertain the degree of contamination of the membrane fraction with other intracellular components, the activities of succinate dehydrogenase and glucose-6-phosphatase were determined in the whole homogenate and in the subcellular fractions by the method of Gibbs and Reimer [26] and Bode et al. [27], respectively.

IV. Materials

Radiolabelled 4,4′-diisothiocyano-2,2′-disulfonic stilbene ([^{3}H]DIDS, specific activity 4 μCi/mmol), obtained from the laboratories of Dr. Aser Rothstein (Hospital for Sick Children, Toronto, Canada), was proven to be chemically pure by nuclear magnetic resonance (NMR) spectroscopy as well as by the criteria of Ship et al. [28]. Non-radioactive SITS, obtained from Gallard-Schlesinger, Long Island, NY and from Peirce Chemical Co., Rockford, IL, proved to be a mixture of at least two substances by NMR spectroscopy. Nevertheless the biological potency of the crude SITS mixture was essentially the same as that of the chemically pure DIDS with respect to its inhibitory

effects on anion transport and on membrane-bound ($Na^+ + K^+$)-ATPase (see Results).

Radioactive ATP compounds, [γ-^{32}P]ATP (specific activity, 9—10 Ci/mmol) and [α-^{32}P]ATP (specific activity, 20—25 Ci/mmol) were obtained from New England Nuclear, Boston, MA. Cyclic AMP compounds (the free acid and potassium salts; the dibutyryl ester; as well as 8-(8-aminooctyl)amino cyclic AMP and 8-benzylthio cyclic AMP) were obtained from ICN, Irvine, CA. Creatine kinase (rabbit skeletal muscle extract), for use in the ATP-regenerating system of the adenylate cyclase assay, was obtained from Sigma, St. Louis, MO. EGTA and EDTA were also obtained from Sigma.

Results

(A) Criteria for the use of radiolabelled disulfonic stilbene(s) as membrane probes

It has been shown that serosally added disulfonic stilbenes (SITS or DIDS) selectively inhibit bicarbonate and chloride reabsorption without appreciably changing sodium reabsorption in short-circuited isolated turtle bladders and that no such effects could be elicited after the mucosal addition of these compounds [12,14]. These findings imply that the disulfonic stilbenes would be useful probes for sites on the anion-selective paths of the basal-lateral membrane if (a) the binding of a disulfonic stilbene to that membrane were covalent or irreversible, and if (b) the disulfonic stilbene did not penetrate the epithelium or gain entry into the cytoplasm. The following experiments yielded data in support of these criteria.

Fig. 1 (a and b) shows the effect of serosally added SITS on the so-called anion-dependent moiety of the short-circuiting current, which is operationally defined here as that across turtle bladders bathed on both surfaces by sodium-free (choline) Ringer solutions containing Cl^- and HCO_3^- (and supplemented with ouabain). Data in the upper panel show that this I_{sc} was reduced to near zero after exposure of the serosal surface to SITS, as has been shown previously [12,14]. Moreover this I_{sc} remained at near-zero levels even after the SITS-containing fluid was removed and replaced with a SITS-free serosal fluid containing 0.5% albumin, a known binder of the disulfonic stilbenes [29,30]. Data in the lower panel show that the prior incorporation of the same quantity of albumin in the serosal fluid was sufficient to protect against the inhibitory effect of susbsequently added SITS, i.e. until the concentration of SITS was increased to more than ten fold that required to produce a maximal inhibition of the anion-dependent I_{sc}. Therefore the effect of SITS (and presumably that of DIDS) on anion transport is irreversible.

In the next set of experiments, the unidirectional fluxes of [^{3}H]DIDS were estimated by the method used previously for other isotopes [2,3]. A small but nonetheless detectable transepithelial flow of [^{3}H]DIDS occurred in the mucosal to serosal direction as well as in the opposite direction (Table I). The magnitude of the serosal to mucosal flux (obtained in the presence of a zero level of anion transport-related current) was about the same as that of the mucosal to serosal flux (obtained in the presence of a control level of this current); and the magnitude of the mucosal to serosal flux was 10^{-6} that of the

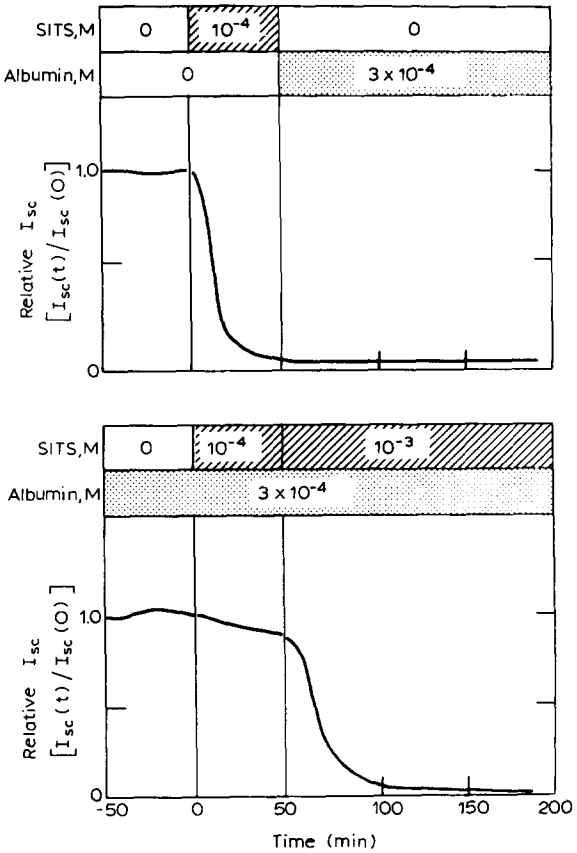

Fig. 1. Relative I_{sc} versus time in two bladders bathed by Na^+-free, HCO_3^--rich Ringer solutions, the exact composition of which is described elsewhere [12]. Relative I_{sc} equals the short-circuiting current at any time (t) after the addition of SITS divided by that at time (0) just before the addition of SITS. Albumin added to serosal fluid (final concentration, 2%) after SITS (upper frame) and before SITS (lower frame).

concomitant I_{sc}. After 4 h of incubation, the maximal concentration of DIDS in the fluid toward which the flux was directed ($3 \cdot 10^{-9}$ M) was no more than $6.6 \cdot 10^{-6}$ of the concentration of DIDS in the fluid from which the flux originated ($4.5 \cdot 10^{-4}$ M).

TABLE I

DIRECTION AND MEAN VALUES FOR THE TRANSEPITHELIAL FLUX OF [^{3}H]DIDS IN SIX SHORT-CIRCUITED HEMIBLADDERS BATHED ON BOTH SURFACES BY IDENTICAL CHOLINE RINGER MEDIA CONTAINING HCO_3^- AND Cl^-

Mean values for mucosal to serosal flux are those calculated from 18 flux periods in three hemibladders and those for serosal to mucosal flux, from 12 periods in three hemibladders.

Transepithelial flux	
Direction	Magnitude (pmol/h per cm^2)
Mucosal to serosal	5.6 ± 0.5
Serosal to mucosal	4.1 ± 0.5

Therefore serosally added disulfonic stilbenes bind irreversibly to but do not appreciably penetrate the basal-lateral membranes. One consequence of this binding is a decrease in anion transport with little or no change in Na^+ transport [12,14,15]. Another consequence (of a short exposure of the serosal surface of the intact bladder to tritiated DIDS) was a preferential radiolabelling of subsequently isolated membrane fraction(s) without decreasing the activity of (Na^+ + K^+)-ATPase or of other enzymes in these membranes (see below).

(B) The mixed membrane fraction

1. Identification of subcellular fractions. Data in Table II, obtained from three separate sets of turtle bladders (ten bladders/set), show that 7% of the total protein in the original homogenate was recovered in the mixed membrane fraction, while the remaining 93% was distributed among the nuclear, mitochondrial, and cytosolic fractions. The specific activity of succinate dehydrogenase, a mitochondrial marker, was maximal in the mitochondrial fraction and minimal in the mixed membrane fraction. The specific activity of carbonic anhydrase, a known cytosolic marker [31], was near zero in both the large and the mixed membrane fractions. The specific activity of glucose-6-phosphatase, a marker for the endocytoplasmic reticulum [32], was lower in the mixed membrane fraction than in the original homogenate. The specific activity of (Na^+ + K^+)-ATPase (a plasma membrane marker) in the mixed membrane fraction was 6—12 fold greater than that in the original homogenate. Finally, the specific binding of [^{3}H]DIDS by the mixed membrane fraction was at least 6—7 fold greater than that by the cytosolic, mitochondrial or nuclear fractions.

In these experiments, some [^{3}H]DIDS, trapped in the interstitial compartment of the bladder wall, was probably eluted into the albumin-free mucosal fluid when the intact epithelial cells were dislodged from the submucosal layer by the EDTA treatment. This contamination, rather than any transmembrane penetration of DIDS, could have accounted for much of the ^{3}H labelling of the cytosolic, mitochondrial and nuclear fractions after the epithelial cells were homogenized. It could be claimed that the same contamination resulted in binding of the disulfornic stilbenes to sites on the inner surface of the basal-

TABLE II

FRACTIONATION OF HOMOGENATE OF ISOLATED TURTLE BLADDER EPITHELIAL CELLS

Yield of total protein and distribution of succinate dehydrogenase (SDH), glucose-6-phosphatase (G-6-Pase), (Na^+ + K^+)-ATPase, and DIDS-binding protein among the following fractions: nuclear, heavy mitochondria, flocculated plasma membranes, and mixed membranes or microsomes.

Epithelial cell fraction	Relative protein (%)	[^{3}H]DIDS binding (cpm/mg)	Enzyme activity (pmol/mg per h)		
			(Na^+ + K^+)-ATPase	G-6-Pase	SDH
Whole homogenate	100	$3.0 \cdot 10^3$	2.3	0.16	1.42
Cytosol	54	$1.6 \cdot 10^3$	0	—	—
Nuclear pellet	22	approx. 10^3	—	—	4.44
Mitochondrial pellet	5	approx. 10^3	—	—	8.54
Flocculated membranes	1	$8.5 \cdot 10^3$	10-20	—	4.50
Mixed membrane pellet	7	10^4	20-30	0.14	0.77

lateral membranes; but this appeared to be unlikely because of the lack of effect of disulfonic stilbenes on the Na^+ transport across the intact bladder [12,14, 15] and because of the degree of inhibition of ($Na^+ + K^+$)-ATPase which was found after the direct addition of the disulfonic stilbenes to the isolated membranes (see below). In view of these technical difficulties, the observed enrichment of the membrane fraction in DIDS-binding protein was considered to be a minimal estimate of enrichment.

Although these data (Table II) do not provide a comprehensive analysis of the distribution of enzymes among the subcellular fractions, they do show that the so-called mixed membrane fraction was minimally contamined (less than 10%) with a known mitochondrial marker (succinate dehydrogenase) or a known cytosolic marker (carbonic anhydrase) and enriched in a known plasma membrane marker, ($Na^+ + K^+$)-ATPase.

This mixed membrane fraction, which probably contains basal-lateral and apical membranes, was then used as follows: (i) to determine the effect of the direct addition of SITS on the activity of ($Na^+ + K^+$)-ATPase in these membranes, in comparison to its effect when added to the serosal surface of the intact bladder; (ii) to determine the effect of the direct addition of norepienphrine and cyclic AMP on the activities of adenylate cyclase and protein kinase, respectively, in these membranes, and finally (iii) to separate the membrane mixture into its component parts by free-flow electrophoresis.

2. Activity of membrane-bound ($Na^+ + K^+$)-ATPase after pretreatment of bladder with disulfonic stilbenes. In this group of experiments, the serosal surfaces of one set of bladders was exposed to SITS while those of a second set (controls) were not so exposed. After a 2 h incubation under these conditions, the mixed membrane fraction of each set was isolated in the manner described above (see Methods).

Pretreatment with SITS produced no decrease but rather an increase in the ($Na^+ + K^+$)-ATPase activity in the subsequently isolated membranes (Table III, first column). These data are in harmony with previously reported data

TABLE III

THE EFFECT OF SITS ON THE ACTIVITY OF ($Na^+ + K^+$)-ATPase IN MIXED MEMBRANE FRACTIONS ISOLATED UNTREATED (CONTROL) AND SITS-TREATED BLADDERS

All values shown are expressed in μmol/mg protein per

Pretreatment of intact bladder	Mixed membrane fraction			
	Incubation conditions	ATPase activity after the following additions		
		None	SITS	Ouabain
None	Mg^{2+}, Na^+, K^+	29.1	15.0	19.9
	Mg^{2+}	18.4	12.5	16.8
	Δ *	10.7	2.5	3.1
SITS	Mg^{2+}, Na^+, K^+	39.1	17.3	28.4
	Mg^{2+}	20.6	12.9	20.1
	Δ	19.1	4.4	8.3

* Δ, the $Na^+ + K^+$-stimulatable moiety of the Mg^{2+}-dependent, membrane-bound ATPase activity.

showing a lack of any effect of disulfonic stilbenes on Na^+ reabsorption across the intact turtle bladder [12,14,15]. Nevertheless SITS was as potent an in vitro inhibitor of the ($Na^+ + K^+$)-ATPase in isolated membranes as was ouabain (Table III). This, together with the lack of effect of SITS or DIDS on the Na^+ transport, suggests that the disfulfonic stilbenes are bound only to sites located in or near anion transport paths on the outer (but not inner) surface of the basal-lateral membranes. If these compounds had penetrated the basal-lateral membranes, one would have expected to see a decrease in Na^+ transport, which did not occur. The reason for the higher ($Na^+ + K^+$)-ATPase activity in membranes isolated from DIDS-treated bladders is not known; it is possible that the recovery of DIDS-treated basal-lateral membranes is greater than that of untreated basal-lateral membranes or that the activity of ($Na^+ + K^+$)-ATPase is increased by the binding of DIDS to the serosal-facing surface of the basal-lateral membrane in the intact bladder.

3. Activities of membrane-bound adenylate cyclase and protein kinase. The next group of experiments was undertaken to look for membrane-bound components that interact with agents which produce changes in ion transport when added to the mucosal fluid, i.e. with norepinephrine and cyclic AMP. In this connection, the mixed membrane fraction was found to contain a norepinephrine-stimulated adenylate cyclase and a cyclic AMP-activated protein kinase (Table V).

Although the specific activity of adenylate cyclase varied widely in going from one to another batch of mixed membranes or large membrane fragments, the norepinephrine-induced increment in this activity (32% on the average) was reproducibility elicited and statistically significant (Table V). The cyclic AMP-induced increment of protein kinase activity in these membranes was also reproducibly elicited and statistically significant (Table V). Not shown are the facts that (i) the basal and the cyclic AMP-stimulated protein kinase activities in membranes from SITS-treated bladders were about the same as those in membranes from control bladders, and that (ii) less than one-third of the cyclic AMP increment of protein kinase activity was eliminated by additions of K^+ or K^+ plus ouabain, which distinguishes the product(s) of this reaction from the phosphorylated intermediates of ($Na^+ + K^+$)-ATPase. Moreover the cyclic AMP-induced increment of this protein kinase activity was elicited in the absence of

TABLE IV

FREE-FLOW ELECTROPHORESIS

Distribution of the cyclic AMP-induced increment of protein kinase (ΔcyclicAMP-PK), the norepinephrine-induced increment of adenylate cyclase (Δnorep-AC) and DIDS-binding proteins in pooled effluent fractions I—IV. The 100% values for ΔcyclicAMP-PK and Δnorep-AC are 183 and 67 pmol/mg per min, respectively, and for DIDS binding, 160 pmol/mg.

Assay	Percentage of maximal activity in free-flow fraction			
	I	II	III	IV
ΔcyclicAMP-PK	<1	0	0	100
ΔNorep-AC	0	0	0	100
DIDS binding	100	65	50	9

exogenously added histones or protamine or other phosphate acceptors, which suggests that the enzyme as well as the associated phosphoryl receptor sites are present in the mixed membrane fraction.

(C) Free flow electrophoresis

In ten experiments on free flow electrophoresis, the mixed membrane fractions were separated into two main groups. One group, which migrated toward the electropositive region of the electric field, contained Mg^{2+}-ATPase, adenylate cyclase and protein kinase; the other, which migrated with a slower mobility in the same electric field, contained $(Na^+ + K^+)$-ATPase and DIDS-binding proteins. Fig. 2, which depicts data from one of these experiments, shows the electrophoretic separation of membranes containing Mg^{2+}-ATPase and adenylate cyclase from those containing $(Na^+ + K^+)$-ATPase. Table IV,

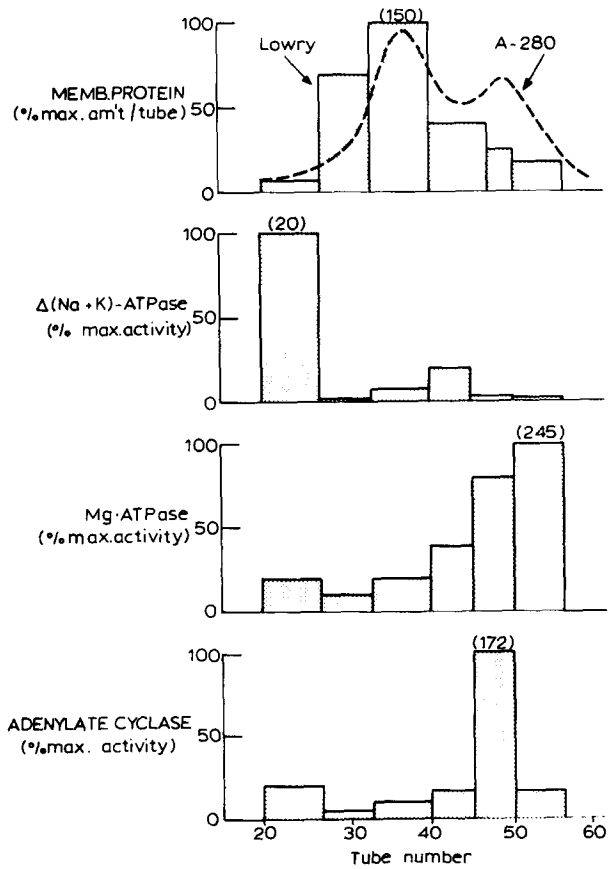

Fig. 2. Electrophoretic separation of the membrane fraction of epithelial cells obtained from a pool of 20 bladders showing the distribution of membrane protein, $(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase and adenylate cyclase among 40 effluent fractions. Numbers in parentheses on top of the columns denote the 100% value of total membrane protein recovered (in mg/effluent tube), the 100% value of the $Na^+ + K^+$ increment and Mg^{2+}-dependent ATPase in μmol/h per mg protein, and the 100% value of adenylate cyclase activity in pmol/min per mg protein.

TABLE V

MEAN VALUES (±S.E.) FOR BASAL AND STIMULATED ACTIVITIES AND PERCENTAGE INCREASES IN THE ACTIVITIES OF NOREPINEPHRINE-SENSITIVE ADENYLATE CYCLASE (UPPER PANEL) AND CYCLIC AMP-SENSITIVE PROTEIN KINASE (LOWER PANEL) IN MIXED MEMBRANE FRACTIONS OF TURTLE BLADDER EPITHELIAL CELLS

Both enzyme activities were measured in paired aliquots of the mixed membrane fractions in six experiments; and protein kinase activity was assayed for in two additional experiments. Data indicate the enzymatic activity in pmol/mg protein per min.

N	Adenylate cyclase			
	Basal	+Norep (10^{-8} M)	%Δ (norep)	$P(\%\Delta = 0)$
6	52.3 ± 11.8	68.7 ± 13.8	32.5 ± 10	<0.002
	Protein kinase			
	Basal	+cyclicAMP (10^{-7} M)	%Δ (cyclicAMP)	$P(\%\Delta = 0)$
8	46.8 ± 6.8	84.3 ± 15.2	85.2 ± 21.0	<0.01

which depicts data from another experiment, shows the electrophoretic separation of membranes containing norepinephrine-sensitive adenylate cyclase and cyclic AMP-sensitive protein kinase from those containing DIDS-binding proteins.

Not pictured here, but depicted in a previous report [11] are data showing the comigration of membranes containing ($Na^+ + K^+$)-ATPase and DIDS-binding proteins. The comigration of the cyclic AMP-sensitive protein kinase and the norepinephrine-sensitive adenylate cyclase, also evident from the data shown in Table VI, is of special significance because the two enzymes were found exclusively in fraction IV and because the incremental specific activities of both enzymes in fraction IV exceeded those of the same enzymes in the original mixed membrane fraction.

Pretreatment of the serosal surface of the intact bladder with [^{3}H]DIDS (at levels which inhibit anion transport but not Na^+ transport) had no detectable effect on the activity and distribution of the enzymatic membrane markers and proteins (other than those which bind DIDS) in the electrophoretically isolated membrane fractions. Thus the activity and distribution of ouabain-sensitive ($Na^+ + K^+$)-ATPase, ouabain-resistant Mg^{2+}-ATPase, norepinephrine-sensitive adenylate cyclase and cyclic AMP-activated protein kinase in electrophoretically isolated membranes from [^{3}H]DIDS-treated bladders (four experiments) was essentially the same as that in membranes from untreated bladders (six experiments).

The interactions of ouabain or norepinephrine with the appropriate enzymes in mixed or electrophoretically separated membranes (Fig. 2 and Tables III–IV) could be correlated with the actions of these agents on ion transport in the intact bladder [6–15]. The inhibitory effect of DIDS or SITS on ($Na^+ + K^+$)-ATPase in the isolated membranes (Table III) contrasts sharply with the lack of effect of the disulfonic stilbenes on Na^+ transport in the intact bladder [12,14]. This suggests that the disulfonic stilbene-binding sites on the basal lateral membrane are discrete and separated from the ouabain-binding sites on the same

TABLE VI

ACTIVITIES OF cyclicAMP-SENSITIVE PROTEIN KINASE (PK) AND NORPINEPHRINE—SENSITIVE ADENYLATE CYCLASE (AC) IN THE MIXED MEMBRANES (MM) AND IN THE ELECTROPHORETICALLY SEPARATED FRACTIONS (I—IV) OF THESE MEMBRANES

Expt. No.	Assay		MM	Enzymatic activity in membrane fraction (pmol/mg per min)			
				I	II	III	IV
1a	PK	Basal	75	158	104	68	112
		+ cyclic AMP	147	159	95	68	295
		Δ	72	1	−9	0	183
1b	AC	Basal	98	34	21	38	254
		+ Norep	110	25	22	31	321
		Δ	12	−9	1	−7	67
2	PK	Basal	17	277	83	166	99
		+ cyclic AMP	23	260	94	163	168
		Δ	5	−17	11	−3	69
3	AC	Basal	35	15	25	32	62
		+ Norep	33	17	24	39	75
		Δ	−2	2	−1	7	13
4	AC	Basal	22	71	38	67	120
		+ Norep	23	68	12	45	172
		Δ	1	−3	−26	−22	52

membrane. The interactions of cyclic AMP with protein kinase in the isolated apical membranes (Tables IV—VI) could not be correlated with or dissociated from ion transport until the latter effect was tested for. This required an additional set of experiments which are described next.

(D) Additional transport experiments

The physiological counterpart of cyclic AMP-activated protein kinase activity in the apical membrane should be a change in the rate of anion transport following addition of cyclic AMP (or one of its derivatives) to the mucosal fluid of the intact bladder. This proved to be the case in a series of tests on cyclic AMP-treated bladders in Na^+-free bathing fluids.

The anion transport-related moiety of short-circuiting current was increased by 50% following the mucosal addition (but not following the serosal addition) of the 8-(8-aminooctyl)amino derivative of cyclic AMP (10^{-4} M) in five experiments and following a similar mucosal addition of the 8-benzylthio derivative of cyclic AMP (10^{-3} M) in two experiments. A smaller but detectable stimulation of anion transport was also found after the mucosal addition of dibutyryl cyclic AMP in two experiments but no effect was elicited after addition of equivalent quantities of the potassium salt or free acid form of cyclic AMP or after the addition of non-cyclic AMP.

In two additional experiments, it was found that the 8-(8-aminooctyl)amino cyclic AMP was without effect on an anion transport that had been increased by the prior mucosal addition of norepinephrine; and conversely, norepinephrine produced no effect on an anion transport that had been increased by the prior addition of amino octyl cyclic AMP.

In summary, the mucosal sidedness of the stimulation of anion transport by cyclic AMP and by norepinephrine in the intact bladder can be construed as physiological counterparts of cyclic AMP-activated protein kinase and norepinephrine-stimulated adenylate cyclase, respectively, in electrophoretically isolated apical membranes. Analogously, the serosal sidedness of the inhibition of Na^+ transport by ouabain and the serosal sidedness of the inhibition of anion transport by the disulfonic stilbenes can be construed as physiological counterparts of ouabain-sensitive ATPase and DIDS-binding protein(s), respectively, in electrophoretically isolated basal-lateral membranes.

Discussion

Free-flow electrophoresis effectively separates a mixed membrane fraction of turtle bladder epithelial cells into two groups of membrane fragments: a basal-lateral group marked by ouabain-sensitive ATPase and high-affinity DIDS-binding protein(s) and an apical group marked by norepinephrine-sensitive adenylate cyclase and cyclic AMP-activated protein kinase. The localization of receptor sites for ouabain and DIDS in basal-lateral membranes corresponds to the serosal sidedness of the action of these agents on ion transport. Similarly the localization of receptor sites for norepinephrine and cyclic AMP in apical membranes corresponds to the mucosal sidedness of the action of these agents on ion transport. The role of each of these membrane elements in ion transport across the turtle bladder is discussed in the following.

Ouabain-sensitive ($Na^+ + K^+$)-ATPase

This membrane marker is probably the Na^+ pump element of the basal-lateral membrane because the concentration of ouabain required for a 50% inhibition of Na^+ transport in the intact bladder is essentially the same as that required for a 50% inhibition of ($Na^+ + K^+$)-ATPase activity in the isolated membrane preparation(s) [6]. Moreover the identity of this enzyme as the Na^+ pump has been directly demonstrated by Goldin and Tong [33] who showed that after the incorporation of ($Na^+ + K^+$)-ATPase (from renal medulla or brain) into synthetic membranes (phospholipid vesicles), Na^+ pumping across these membranes is induced in the presence of ATP, Mg^{2+}, Na^+, and K^+ and inhibited by ouabain.

DIDS-binding proteins

A tentative identification of the DIDS-binding proteins as carrier elements in the anion-selective path of the basal-lateral membrane can be deduced from the following facts: (i) DIDS receptor sites (of relatively high binding 'affinity') are detectable only in basal-lateral membranes isolated from bladders in which [^{3}H]DIDS had been added to the serosal fluid to block anion transport. (ii) Such sites are not found in either the basal-lateral or apical membranes isolated from bladders in which [^{3}H]DIDS had been added to the mucosal fluid; in this case, anion transport is not blocked [12,14].

A more detailed identification of DIDS-binding proteins as discrete anion carriers comes from data on erythrocytes. The band III proteins of erythrocyte membranes, isolated in the 95 000 dalton region of a polyacrylamide gel, con-

tain the DIDS-binding protein and ($Na^+ + K^+$)-ATPase. After these proteins are treated with pronase, the DIDS-binding protein migrates to the 65 000 dalton region while the ($Na^+ + K^+$)-ATPase remains at the 95 000 dalton region, which suggests that Na^+ pump sites are separable from the anion exchanger sites [29,30]. The discreteness of these pump and exchanger sites is consistent with the fact that the number of DIDS-binding sites/red blood cell is orders of magnitude higher than the number of ($Na^+ + K^+$)-ATPase sites [29]. In the turtle bladder however, the number of DIDS-binding sites is probably less than that of ouabain-binding (Na^+ pump) sites on the basal-lateral membrane, in view of the fact that the rate of net Na^+ transport is 10—20-fold greater than that of anion transport [1—5].

Norepinephrine-sensitive adenylate cyclase

This enzyme marks the electrophoretically defined apical membrane fragments, which were physically separated from the basal-lateral membranes. Although it is tempting to postulate that the anion transport in vivo is under the control of norepinephrine which acts as a first messenger to stimulate a membrane-bound adenylate cyclase, consideration of the present data gives rise to some disquieting problems: (i) The alleged first messenger (norepinephrine) apparently acts on the mucosal rather than on the serosal surface of the bladder epithelial cell layer. This is opposite to a more conventionally conceived and observed situation in the proximal renal tubule where norepinephrine apparetly acts only from the blood side to induce an increased rate of fluid reabsorption [34]. (ii) The concentration of mucosal norepinephrine required for the acceleration of anion transport (10^{-5}—10^{-4} M) in the intact bladder is orders of magnitude greater than that required for a maximal stimulation of adenylate cyclase (10^{-8} M) in the isolated apical membranes. Therefore this stimulated increment of adenylate cyclase activity is a useful marker for the apical membrane; but this does not establish a possible physiological role of this neurotransmitter in the regulation of anion transport. (iii) The apical membrane localization of this norepinephrine-sensitive adenylate cyclase in the turtle bladder contrasts sharply with the basal-lateral membrane localization of two other adenylate cyclases in the mammalian kidney: one stimulated by parathormone, is found in the proximal tubule region [35] and another, stimulated by vasopressin is found in the collecting duct region [36]. At this stage, data on the turtle bladder are sufficient to show that the enzyme (norepinephrine-stimulated adenylate cyclase) is a useful marker for the apical membrane, but not yet sufficient to show that the neurotransmitter (norepinephrine) is the first messenger in the regulation of anion transport.

Cyclic AMP-activated protein kinase

Whereas this enzyme, like norepinephrine, is a useful marker of the apical membrane, the present data are not sufficient to establish the role of cyclic AMP-dependent phosphorylation in the regulation of anion transport in vivo. The concentration of cyclic AMP required for inducing a stimulation of the anion transport current in the intact bladder (10^{-3}—10^{-4} M) is orders of magnitude greater than that required for inducing an increment of protein kinase activity (10^{-6}—10^{-8} M) in the isolated apical membranes. This might be due to

the possibility that cyclic AMP has to be generated within the cytoplasm and has to act upon the cytoplasmic rather than upon the external surface of the luminal membrane. Moreover the amount of cyclic AMP that can be driven into the cytoplasm of the in vivo epithelium could be quite small because of a low cyclic AMP permeability of both plasma membranes and because of the electronegativity of the cell fluid with respect to the mucosal fluid.

Current status and problems

Table VII is a summary of the current state of knowledge on transmembrane ion flows across intact turtle bladder epithelial cells and on the corresponding elements in membrane fragments isolated from these cells. The lack of identification of the putative amiloride-binding element in the apical membrane is due to the reversibility of the action of amiloride in the intact system and the lack of any known amiloride-sensitive activity in the isolated membranes. The lack of identification of the anion pump elements, an absolute deficiency in the current knowledge, poses a problem in the interpretation of basic data on anion transport across the intact epithelium. Whereas the existence of anion-dependent ATPase in plasma membranes has been challenged [37] and defended [38], its role in anion or proton transport has not yet been established. The presence of a K^+-stimulated ATPase and related K^+/H^+ exchange processes in the plasma membranes of gastric parietal cells [39,40] has yet to be demonstrated in non-gastric epithelia including the turtle bladder.

Compounding the complexity of these problems is the current uncertainty on the nature of the process of active Cl^- transport; some postulating that this is mediated by an electroneutral process of HCO_3^- secretion in exchange for Cl^- reabsorption [41] and others postulating that Cl^- reabsorption and HCO_3^-

TABLE VII

SUMMARY OF KNOWLEDGE AND HYPOTHESES ON TRANSMEMBRANE ION FLOWS ACROSS INTACT TURTLE BLADDER EPITHELIAL CELLS

Ion-selective paths deduced from data on intact epithelium	Corresponding transport-related elements in	
	Apical membrane	Basal-lateral membrane
Sodium reabsorption Transapical (passive); trans basal-lateral (active); presumably electrogenic	Amiloride-sensitive element (not yet isolated)	Ouabain-sensitive (Na + K)-ATPase
Bicarbonate reabsorption Transapical/(active); presumably electrogenic; trans basal-lateral (passive)	Pump element unknown, but coupled to norepinephrine-sensitive adenylate cyclase and cyclicAMP-sensitive protein kinase	DIDS-binding elements; acetazolamide-sensitive element (not yet isolated)
Chloride reabsorption Transapical (active); electrogenicity, ±; trans basal-lateral (passive)	Pump element (unknown and coupling to cyclase and kinase elements no yet ascertained)	DIDS-binding elements; acetazolamide sensitive element (not yet isolated)

secretion are each mediated by an electrogenic pump [2,42]. In addition, there is no agent that interacts directly with the anion pumps in the way that ouabain interacts with the Na^+ pump. The norepinephrine-sensitive adenylate cyclase and the cyclic AMP-sensitive protein kinase in the apical membranes are tentatively considered to be regulatory elements rather than pump elements for the active transport of Cl^-, HCO_3^- (or protons) across the bladder cell. Finally, it should be noted that there is no unique evidence for the presence of Cl^-/HCO_3^- exchangers (active or passive), even though the action of disulfonic stilbenes might be taken as tentative evidence for the existence of such elements in turtle bladder as well as in erythrocyte membranes.

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