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INHIBITORY EFFECT OF ACETAZOLAMIDE ON THE ACTIVE CHLORIDE AND BICARBONATE TRANSPORT MECHANISMS ACROSS SHORT-CIRCUITED TURTLE BLADDERS

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SUMMARY

Acetazolamide added to the serosal fluid, at a final concentration of 0.1 mM, inhibits the active transport of Cl^- and HCO_3^- across the short-circuited turtle bladder. When bladders are bathed on both mucosal and serosal surfaces by Na^+-free choline Ringer solutions containing Cl^- and HCO_3^-, the net Cl^- flux (m to s) accounts for only half of the short-circuiting current. If HCO_3^- is removed from the mucosal fluid the net Cl^- flux accounts for all of the short-circuiting current. In the experiments presented in this report both the net Cl^- flux and the short-circuiting current were inhibited by acetazolamide. When Cl^- was replaced by SO_4^{2-} in the Na^+-free choline Ringer the short-circuiting current associated with the presence of HCO_3^- in the bathing fluids was also inhibited by addition of acetazolamide to the serosal fluid. These inhibitory effects could be reversed in most of the experiments.

Previous work has shown that ouabain-treated bladders in Na^+-rich ambient media were indistinguishable from untreated bladders in Na^+-free (choline) ambient media. This finding was confirmed. In this connection, acetazolamide inhibited, in the same quantitative and qualitative fashion, the active transport of Cl^- and HCO_3^- in ouabain-treated Na^+-rich preparations as well as in Na^+-free preparations.

Acetazolamide fails to inhibit both moieties, the Mg^{2+}-dependent and the $(Na^+ + K^+)$-stimulated, of the ATPase activity found in the microsomes of the turtle bladder.

A relation between the active transport of Cl^- and HCO_3^- and the enzyme carbonic anhydrase is difficult to entertain at this point since homogenates of the turtle bladder do not possess carbonic anhydrase activity.

INTRODUCTION

The bladder of the turtle *Pseudemys scripta* actively transports Na^+ (refs. 1–7) and Cl^- (refs. 3, 4, 8) from the mucosal (m) to the serosal (s) fluid. It also acidifies the mucosal fluid by either removal of HCO_3^- (refs. 9, 10) or possibly by secretion of H^+ (refs. 6, 9, 11). When bladders are bathed in Na^+-rich Ringer, the serosal surface

Abbreviation: PD, potential difference.

becomes electropositive with respect to the mucosal surface by 50–90 mV, and short-circuiting is achieved by passing positive current from mucosa to serosa. However, when bladders are bathed in Na^+-free choline Ringer, the Na^+ transport vanishes, the serosal surface becomes electronegative with respect to the mucosal surface by 20–70 mV and short-circuiting is achieved by passing positive current from serosa to mucosa[4,8].

In bladders bathed by Na^+-free, HCO_3^--rich media and under short-circuiting conditions, Cl^- and HCO_3^- transport account for all of the short-circuiting current,

$$I_{sc} = I_{Cl^-} + I_{HCO_3^-}$$

where I_{sc} denotes positive current from m to s. The current density, $I_{Cl^-} + I_{HCO_3^-}$, reaches the same value in the Na^+-free system as it does in the Na^+-rich system[4,8].

The active transport (m to s) of Cl^- has been demonstrated by measuring Cl^- concentration changes chemically in sac preparations bathed by HCO_3^--rich Na^+-free Ringer solutions[8] and by measuring radioactive Cl^- fluxes in short-circuited preparations bathed by HCO_3^--rich Ringer solutions with and without Na^+ (refs. 4, 8).

The active transport of HCO_3^- (m to s), rather than of H^+ (s to m) has been established directly on the basis of finding simultaneous decreases of pH, HCO_3^-, and pCO_2 in the mucosal fluids of sacs bathed by sodium Ringer containing bicarbonate[9,10]. Acid secretion has been demonstrated in bladders bathed by HCO_3^--free solutions. This acidification process could be due to the same HCO_3^- reabsorption mechanism proposed above[9,10] or it could be due to a different process, a H^+ secretion as has been suggested by SCHILB AND BRODSKY[9] and claimed by STEINMETZ[11] studying the acidification of mucosal fluids devoid of HCO_3^-. STEINMETZ *et al.*[6] also found that acetazolamide, a known carbonic anhydrase inhibitor, reduces significantly the acidification process of bladders bathed in HCO_3^--free solutions.

Work to be presented deals with the effect of acetazolamide on the active transport of ions when bladders are bathed by Na^+-free choline Ringer solutions containing HCO_3^-.

The present study will demonstrate that: (1) acetazolamide inhibits the active transport of both Cl^- and HCO_3^- in the turtle bladder, a tissue devoid of carbonic anhydrase activity[12]; (2) acetazolamide reduces the transport rate of Cl^- and HCO_3^- in ouabain-treated bladders bathed in Na^+-rich Ringer solutions; (3) acetazolamide does not change Mg^{2+}-dependent and $(Na^+ + K^+)$-stimulated moieties of the ATPase activity found in the microsomal fractions of mucosal cells of the turtle bladder.

MATERIALS AND METHODS

Elution and mounting

Urinary bladders of the turtle *Pseudemys scripta* were excised, immersed and dissected in Na^+-free choline Ringer. Blood was massaged out of the cut ends of the peritoneal blood vessels. The bladder was incubated for a period of 45 min in Na^+-free Ringer with the object of eluting the Na^+ present in the tissue.

The tissue in the form of a flat sheet was mounted in an Ussing-type Lucite chamber[13], modified by REHM[14], and interposed between appropriate Ringer solutions. Two types of chambers were used. Part of the experiments were performed in a double-barreled chamber which has been described previously[8] and which provided

an exposed bladder area of 1.5 cm^2. Most of the experiments were performed in a single-barreled Lucite chamber, with an area of 5 cm^2. The structure and function of this chamber as well as the circulation of the ambient fluid is the same as that of the double chamber[8]. The use of the single-barreled chamber, with larger exposed area, did not permit the study of paired halves of a given bladder. On the other hand, because of the larger exposed area, the measured transport parameters were magnified, and thus, more accurate measurements could be made. This was particularly desirable in the case of the transport of Cl^- and HCO_3^-, since the rate of transport of these ions is not as high as the rate of transport of Na^+.

Electrical and flux measurements

The tissue was maintained short-circuited by using the technique of USSING AND ZERAHN[13]. Electrical measurements of potential difference (PD), I_{sc} and R were performed as described previously[8]. Values for the short-circuiting current were recorded continuously, except when the external circuit was opened for 1 sec at 5-min intervals to obtain values for the spontaneous transbladder potential.

Unidirectional Cl^- fluxes were measured after addition of $^{36}Cl^-$ (Oak Ridge, Tenn.) in the form of choline Cl^- as described previously[8].

In experiments where the area of the bladder exposed to the bathing solutions was 5 cm^2 dry tissue weight was 79.8 $\pm$ 22.6 mg and tissue water 643 $\pm$ 158 μl.

All experiments were performed at 22–25°.

Solutions and analysis

The composition of each bathing solution used herein in terms of mM concentrations was as follows:

(a) HCO_3^--rich choline Ringer: $Choline^+$, 101; Cl^-, 92; K^+, 4.8; Ca^{2+}, 2.0; $H_2PO_4^-$, 0.07; HPO_4^{2-}, 0.73; HCO_3^-, 17; Mg^{2+}, 0.8; SO_4^{2-}, 0.8; CO_2, 0.33; and glucose, 11. Final osmolality 220 mosM. The final pH varied from 7.4 to 7.6 in different experiments.

(b) HCO_3^--rich choline SO_4^{2-} Ringer: This solution was designed to substitute the two transportable ions Na^+ and Cl^-. This meant that the only known transportable ion present was HCO_3^-. $Choline^+$, 118.1; SO_4^{2-}, 92.8; Ca^{2+}, 2.0; $H_2PO_4^-$, 0.07; HPO_4^{2-}, 0.73; HCO_3^-, 17; Mg^{2+}, 0.8; CO_2, 0.33; and glucose, 11. Sucrose was added to achieve a final osmolality of 220 mosM. The final pH was kept between 7.4 and 7.6 pH units.

(c) HCO_3^--rich NaCl Ringer: Na^+, 101; Cl^-, 92; K^+, 4.8; Ca^{2+}, 2.0; $H_2PO_4^-$, 0.07; HPO_4^{2-}, 0.73; HCO_3^-, 17; Mg^{2+}, 0.8; SO_4^{2-}, 0.8; CO_2, 0.33; and glucose, 11. Final osmolality 220 mosM. The final pH varied from 7.4 to 7.6 pH units.

Chemical measurements of electrolyte concentration, pH and osmolality were performed routinely on all ambient fluids by techniques described previously[18].

When in contact with the bladder, the measured pH of the Ringer solution was kept constant by continuous gassing with O_2–CO_2 (99:1, v/v).

Sources of material

Turtles were obtained from Lemberger Co., Oshkosh, Wisc., U.S.A. Experiments were performed between the months of May and October.

Sodium methyl sulfate was obtained from Eastman Organic Chemicals, Ro-

chester, N.Y. Ouabain (G-strophanthidin), Tris–ATP, ATP (disodium salt), L-histidine·HCl, and imidazole Grade I, were obtained from Sigma Chemical Co., St. Louis, Mo. Acetazolamide (Diamox) was kindly supplied by Lederle Laboratories.

Preparation of tissue and method of procedure for assay of ATPase activity

Isolated mucosal cells were obtained and microsomal fractions separated as described in a previous report[7]. In the assay for ATPase, the composition of the reaction mixture, expressed in terms of final mM concentration was: Tris–ATP or ATP (sodium salt), 2.0; $MgCl_2$, 2.0; EDTA, 0.2; imidazole·HCl, 40; histidine·HCl, 40; NaCl, 96; and KCl, 24. Protein enzyme concentration was 10–20 μg/ml and the final volume of the flask was 5 ml. Acetazolamide, at a final concentration of 0.1 mM, was added to tubes in which both moieties of ATPase were assayed. Tubes with and without ouabain were used to assay both Mg^{2+}-dependent ATPase activity and $(Na^+ + K^+ + Mg^{2+})$-ATPase or total activity, respectively. After 5 min of pre-incubation, the reaction was started by addition of ATP and incubated at 38° for 10 min. The reaction was stopped by addition of 5 ml of cold 6% $HClO_4$. Control tubes, carried through all incubations, were of two types: those without the ATP and those without the enzyme. Aliquots of the final mixture were analyzed for P_i by the method of BERENBLUM AND CHAIN[16]. Protein concentration was determined by the method of LOWRY *et al.*[17].

RESULTS

Effect of acetazolamide on the Cl^- transport

In the first set of experiments, bladders were mounted as flat sheets in the Lucite chamber and interposed between identical Na^+-free choline Ringer solutions containing Cl^- and HCO_3^-. The pH was kept fixed at about 7.6 by gassing both mucosal and serosal fluids with O_2–CO_2 (99:1). Samples for flux measurements were taken starting 70 min after addition of the radioactive Cl^- to the bathing fluid, and continued every 30 min throughout the experiment. Acetazolamide, at final concentrations of 0.1 mM, was added to the serosal fluid. 10–15 min after the addition of the drug,

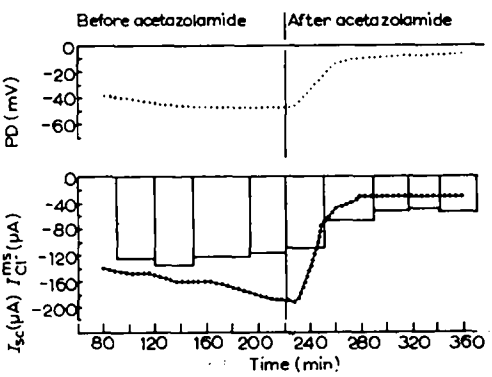

Fig. 1. PD, (m to s) flux of Cl^- ($I^{ms}_{Cl^-}$) and short-circuiting current (I_{sc}) as a function of time before and after addition of 0.1 mM acetazolamide to the serosal fluid. Bladder bathed by choline Ringer containing Cl^- and HCO_3^-.

both short-circuiting current and PD started to decline steadily. It took about 1 h until the transport parameters achieved a new steady state, which lasted without major change until the end of the experiment. The duration of the experiment was 350 min. Acetazolamide was added at time 160–180 min.

Electrical and flux measurements taken 90 min before the addition of acetazolamide were labelled "period before acetazolamide" while measurements starting 1 h after addition of the drug and continuing until the end of the experiment were labelled "period after acetazolamide".

Fig. 1, a plot of values of m to s flux of Cl^- ($I_{Cl^-}^{ms}$), short-circuiting current (I_{sc}) and PD *versus* time in one of the experiments to be presented in Table I, illustrates the time course and magnitude of the action of acetazolamide on the three parameters measured. Note the parallelisms of the change of both I_{sc}, and PD, starting 15 min after addition of the drug. Cl^- flux changes lagged behind those of the electrical parameters. 1 h after addition of the inhibitor, the rate of both I_{sc} and $I_{Cl^-}^{ms}$ remained almost unchanged throughout the rest of the experiment.

TABLE I

EFFECT OF ACETAZOLAMIDE ON THE Cl^- AND HCO_3^- TRANSPORT PARAMETERS

Electrical parameters and unidirectional (m to s) flux of Cl^-, before and after addition of 0.1 mM acetazolamide to the serosal fluid in 5 experiments. Bladders were bathed by choline $Cl^- - HCO_3^-$ containing Ringer gassed with $O_2 - CO_2$ (99:1, v/v). Extensive parameters are expressed per 5 cm^2 area of tissue. Values are ± S.E.

Period	*PD* (*mV*)	I_{sc} (μA)	*R* (Ω)	$I_{Cl^-}^{ms}$ (μA)
Before	−53.6 ± 6.0	−208 ± 54.3	304 ± 56.8	141 ± 26.1
After	−16.2 ± 2.7	− 55.0 ± 11.2	294 ± 36.1	48.4 ± 4.2
Mean % difference*	−70.1	− 71.6	−0.70	−60.2
Probability*	$P < 0.001$	$P < 0.001$	$P > 0.9$	$P < 0.001$

* Values for individual changes before and after addition of acetazolamide.

Table I presents mean values for the electrical parameters and m to s Cl^- fluxes, before and after addition of acetazolamide at a final concentration of 0.1 mM to the serosal fluid under experimental conditions similar to those described for the experiment of Fig. 1. Mean per cent differences and *P* values are given at the bottom of the table. It can be seen that acetazolamide significantly inhibited the m to s flux of Cl^-, I_{sc} and PD. Values of d.c. resistance, before and after the addition of the drug, were not significantly different.

Table II, similar in format to Table I, presents data from a different set of experiments, obtained under the same experimental conditions as the experiments of the previous table, except that unidirectional Cl^- fluxes were measured from s to m. It can be seen that although I_{sc} and PD were inhibited after the addition of acetazolamide, there was no significant change in the unidirectional Cl^- fluxes measured from s to m. Mean values, mean per cent differences and probability for I_{sc}, PD and *R* before and after addition of acetazolamide were similar to those found in the set of bladders presented in Table I.

TABLE II

EFFECT OF ACETAZOLAMIDE ON THE Cl^- AND HCO_3^- TRANSPORT PARAMETERS

Electrical parameters and unidirectional (s to m) flux of Cl^-, before and after addition of 0.1 mM acetazolamide to the serosal fluid in 5 experiments. Bladders were bathed by choline $Cl^- - HCO_3^-$ containing Ringer gassed with $O_2 - CO_2$ (99:1, v/v). Extensive parameters are expressed per 5 cm^2 area of tissue. Values are mean ± S.E.

Period	*PD* (*mV*)	I_{sc} (μA)	*R* (Ω)	$J^{sm}_{Cl^-}$ (μA)
Before	−59.5 ± 6.2	−171 ± 24.6	355 ± 28.2	33.6 ± 4.6
After	−22.0 ± 4.0	− 64.0 ± 17.0	372 ± 37.9	36.9 ± 5.0
Mean % difference*	−62.7	−63.5	0.42	8.1
Probability*	$P < 0.001$	$P < 0.001$	$P > 0.6$	$P > 0.3$

* Values for individual changes before and after addition of acetazolamide.

The properties and components of the s to m unidirectional flux of Cl^- in the turtle bladder have not been characterized. It is not known if it can be accounted for, in its entirety, by free diffusion or if there is a component of exchange diffusion. Whatever may be the case, this unidirectional flux was not changed by the addition of acetazolamide. Similarly, the backflux or secretory to nutrient flux of Cl^- in the frog stomach[18] was not affected by acetazolamide in any of its components—free diffusion or exchange diffusion.

The findings of Table I and Table II indicate that acetazolamide, as in the case of the frog stomach, inhibits the m to s unidirectional flux of Cl^- without affecting the s to m flux.

Table III presents mean values of short-circuiting current and net chloride flux, combining data of Tables I and II. It can be seen that acetazolamide had a very marked inhibitory effect on PD and short-circuiting current. Part of the decrease observed in the short-circuiting current was due to the decrease in the net Cl^- flux.

TABLE III

EFFECT OF ACETAZOLAMIDE ON SHORT-CIRCUITING CURRENT AND NET FLUX OF Cl^-

Mean values for short-circuiting current (I_{sc}), net chloride flux ($I^{net}_{Cl^-}$) and undetermined portion of the short-circuiting current ($I_{sc} - I^{net}_{Cl^-}$), combining data of Tables I and II.

Period	I_{sc} (μA)	$I^{net}_{Cl^-}$ (μA)	$I_{sc} - I^{net}_{Cl^-}$ (μA)
Before	−191	−107	−84
After	− 59	− 11.5	−47.5

Effect of time in control experiments

From previous study in the isolated turtle bladder[4,8], it has been recognized that, in general, transport processes have a tendency to decay with time. This is specially the case for the transport of Cl^- and HCO_3^-. In order to ensure that the decreases in the ion transport rate found after addition of acetazolamide were not due

to aging of the preparation *in vitro*, control runs were performed in the same batch of turtles as that used for testing the acetazolamide effect. These experiments were designed to have the same time-course as those in which acetazolamide was added. Since acetazolamide was found to inhibit the m to s flux of Cl^- and did not affect the s to m flux, the control experiments included measurements of the m to s flux of Cl^-.

TABLE IV

EFFECT OF TIME IN CONTROL EXPERIMENTS

Electrical parameters and unidirectional (m to s) flux of Cl^- in four control bladders bathed by choline $Cl^- - HCO_3^-$-rich Ringer gassed with $O_2 - CO_2$ (99:1, v/v). No inhibitor was added. Periods before and after refer to time periods corresponding to those labelled before and after acetazolamide in previous tables. Extensive parameters are expressed per 5 cm^2 area of tissue. Values are mean ± S.E.

Period	*PD* (*mV*)	I_{sc} (μA)	*R* (Ω)	$I_{Cl^-}^{ms}$ (μA)
Before	−50.5 ± 6.6	−295 ± 80.7	211 ± 56.9	202 ± 60.7
After	−41.2 ± 9.7	−225 ± 69.2	207 ± 39.4	185 ± 50.4
Mean % difference*	−21.2	−23.2	−4.5	−1.5
Probability*	$P < 0.1$	$P < 0.2$	$P < 0.5$	$P < 0.9$

* Values for individual changes due to aging.

Table IV, similar in format to the previous tables, shows mean values for $I_{Cl^-}^{ms}$, I_{sc}, PD and resistance in time periods that corresponded to those labelled before and after acetazolamide in Tables I and II. Mean per cent differences between period labelled before and after were not significantly different from each other.

It can be seen that the time-dependent change of the preparation *in vitro*, although present in almost all the experiments, was small compared with the acetazolamide-induced changes. This can be proven comparing the mean per cent changes obtained in both cases. In all measured parameters the mean per cent changes after acetazolamide were significantly different from those changes due to time alone, the only exception being the d.c. resistance.

The values of the extensive variables (I_{sc}, I_{Cl^-} and R^{-1}) during the period before acetazolamide were greater than those obtained previously in bladders bathed in the same Na^+-free ambient fluids[8]. This was expected since the exposed area of the bladder was 5 cm^2 in this report, and 1.5 cm^2 in the previous report[8].

Effect of acetazolamide on the HCO_3^- transport

As has been pointed out previously, when HCO_3^- is present in the mucosal fluid, the net flux of Cl^- ($I_{Cl^-}^{net}$) does not account for all of the short-circuiting current. This undetermined portion of the short-circuiting current ($I_{sc} - I_{Cl^-}^{net}$), which amounts to about 50 % of the total, has been interpreted to be due to the active transport of HCO_3^- (refs. 4, 8). Data on Table III show the amount of the undetermined portion of the short-circuiting current, labelled ($I_{sc} - I_{Cl^-}^{net}$), before and after the addition of acetazolamide. It can be seen that the value of ($I_{sc} - I_{Cl^-}^{net}$) after acetazolamide was inhibited by about 49 %.

In order to clarify this last finding, a new set of experiments was designed. Bladders were bathed on both surfaces by choline Ringer in which Cl^- was replaced by SO_4^{2-}. This meant that the only known transportable ion present in the bathing fluids was HCO_3^-. Concentrations of other constituents of the Ringer remained unchanged (see MATERIALS AND METHODS). The solutions were gassed with 99% O_2 + 1% CO_2.

TABLE V

EFFECT OF ACETAZOLAMIDE ON THE HCO_3^- TRANSPORT PARAMETERS

Electrical parameters before and after addition of 0.1 mM acetazolamide to the serosal fluid in 5 experiments. Bladders were bathed by HCO_3^--rich choline SO_4^{2-} Ringer gassed with O_2–CO_2 (99:1, v/v). Extensive parameters are expressed per 5 cm^2 area of tissue. Values are mean ± S.E.

Period	*PD* (*mV*)	I_{sc} (μA)	*R* (Ω)
Before	−35.6 ± 6.7	−83.4 ± 7.8	412 ± 44.2
After	− 9.2 ± 2.7	−27.4 ± 5.1	334 ± 36.6
Mean % difference*	−73.6	−67.5	−16.2
	− 2.9	− 4.3	− 3.8
Probability*	$P < 0.001$	$P < 0.001$	$P < 0.01$

* Values for individual changes before and after addition of acetazolamide.

Table V shows mean values for I_{sc}, PD and *R* obtained in bladders bathed by HCO_3^--rich choline SO_4^{2-} Ringer, before and after addition of acetazolamide to the serosal fluid. The time-course of the experiment was kept exactly as the time-course designed for experiments of Fig. 1 and Tables I and II. It can be seen that the drug inhibited the short-circuiting current by 67.5% which is somewhat higher than the percent inhibition obtained by using the calculated values of Table III. The mean values of PD before acetazolamide were also significantly different from those obtained after the addition of the drug. In contrast to the constancy of resistance in the previous experiments, the resistance decreased significantly after acetazolamide in the series of experiments of Table V.

All changes produced by acetazolamide were reversed when the drug was removed from the serosal fluids. This reversible type of behavior was elicited in eight out of ten experiments. In the two remaining experiments, acetazolamide induced progressive and sustained decreases in I_{sc} and I_{Cl^-} that were not reversed when the drug was removed.

Effect of removal of Cl^- from ambient fluids on electrical parameters

Beside the effect of acetazolamide, it is interesting to note two additional findings in comparing data on electrical parameters in Tables I, II and IV, with those in Table V.

The mean values for PD and I_{sc} obtained when Cl^- was present in the Ringer solution (53.6 mV and 208 μA) were about twice those obtained when Cl^- was not present in the Ringer solution (35.6 mV and 83.4 μA); and these differences were statistically significant ($0.02 < P < 0.05$). This suggests that the short-circuiting

current and the instantaneous transbladder potential difference are a reflection of the rate of transport of both Cl^- and HCO_3^-.

Another observation was that the flow of HCO_3^- estimated from the difference current, $I_{sc} - I_{Cl^-}^{net}$, in the presence of Cl^- and HCO_3^- (84 μA) was practically the same as the directly measured current, I_{sc}, in the presence of HCO_3^-, but not Cl^- (83.4 μA). (Compare periods before acetazolamide in Tables III and V.) This finding suggests that the rate of transport of HCO_3^- did not vary in the presence or absence of Cl^-; and that it remained constant despite the changes in ionic strength occasioned by replacing Cl^- with SO_4^{2-}.

Effect of acetazolamide in ouabain-treated bladders

It has been shown that ouabain added to the serosal fluid reduces the net transport of Na^+ to approximately zero[7]. When Cl^- and HCO_3^- are present in the mucosal fluid, ouabain causes a reversal of orientation of I_{sc} and PD such that the serosa becomes electronegative to the mucosa. This finding is consistent with the continued active transport of Cl^- and HCO_3^-. The purpose of the next set of experiments was to determine the effect of acetazolamide on the reversed short-circuiting current and on the m to s flux of Cl^-.

Bladders mounted in double-barrelled Lucite chambers were bathed by Na^+ Ringer solutions containing Cl^- and HCO_3^-. Isotopic Cl^- fluxes and electrical parameters were measured. Ouabain was added to the serosal fluid of both experimental and control half-bladders. After 60 min of incubation I_{sc} had decreased, reversed in orientation and reached steady levels. After three control periods of Cl^- flux measure-

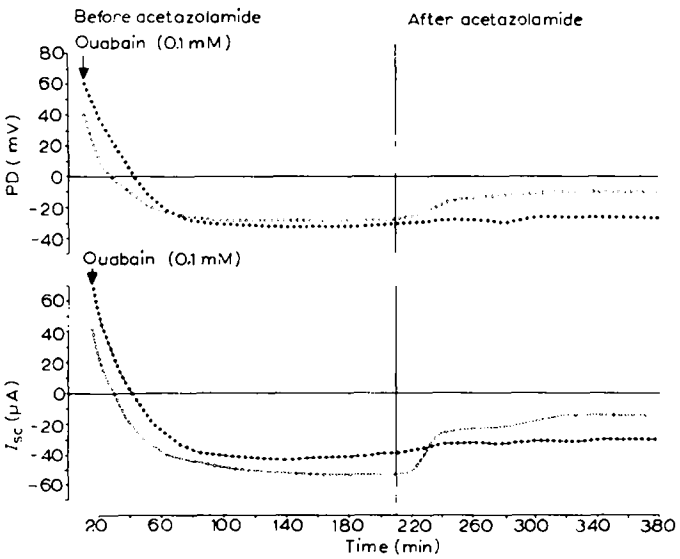

Fig. 2. PD and I_{sc} as a function of time in paired hemibladders. ○—○, experimental; ●—●, control. Both tissues were bathed by Na^+ Ringer. At the beginning of the experiment, ouabain was added to the serosal fluid of both hemibladders. At t = 210 min acetazolamide was added to the serosal fluid of the experimental half. Note the progressive decrease in PD and I_{sc} after addition of the sulfonamide. There was a slight time-dependent decay in the parameters of the control bladder.

TABLE VI

EFFECT OF ACETAZOLAMIDE IN OUABAIN-TREATED BLADDERS

Mean values for $I_{Cl^-}^{ms}$, I_{sc}, and PD in six paired hemibladders before and after addition of acetazolamide to the serosal fluid of the experimental, but not to that of the control hemibladder. Both hemibladders were bathed by Na^+ Ringer, containing Cl^- and HCO_3^-. Ouabain 0.1 mM was added to both control and experimental bladders. The difference in the average value of each parameter before and after addition of acetazolamide was obtained for each individual experimental and control half-bladders. These differences (after *minus* before) in each experimental half less those in each paired control half were designated $\Delta(\Delta I_{Cl^-}^{ms})$, $\Delta(\Delta I_{sc})$ and $\Delta(\Delta PD)$, thus providing statistically analyzable data corrected for the time-dependent effects. Extensive parameters are expressed per 1.5 cm^2 area of tissue. Values are means of 6 experiments.

Period	$I_{Cl^-}^{ms}$ (μA)		I_{sc} (μA)		*PD* (*mV*)	
	Experimental	*Control*	*Experimental*	*Control*	*Experimental*	*Control*
Before	41.9	34.6	−56.5	−49.0	−38.5	−35.5
After	20.2	29.2	−16.5	−40.2	−11.7	−25.3
	$\Delta(\Delta I_{Cl^-}^{ms})$		$\Delta(\Delta I_{sc})$		$\Delta(\Delta PD)$	
Mean ± S.E.	−16.4 ± 4.9		−31.2 ± 6.6		−16.7 ± 4.3	
Probability	$P < 0.05$		$P < 0.01$		$P < 0.02$	

ments, 0.1 mM acetazolamide was added to the serosal fluid of the experimental half, but not to the control half.

Fig. 2 is a plot of I_{sc} and PD *versus* time in a pair of half-bladders before and after addition of acetazolamide to the experimental half, but not to the mated control half-bladder. 10 min after addition of acetazolamide the characteristic progressive decrease in I_{sc} and PD occurred as is illustrated in the figure.

Table VI shows mean values and statistical parameters for I_{sc}, $I_{Cl^-}^{ms}$, PD and resistance obtained in six experiments designed similarly to that of the experiment shown in Fig. 2.

As was the case in the Na^+-free system, there occurred decreases in $I_{Cl^-}^{ms}$, I_{sc} and PD after addition of acetazolamide in the experimental half-bladders which had been pre-treated with ouabain and bathed in Na^+-rich media. Time-dependent changes in control halves were negligible compared to the drug-induced changes in the experimental half-bladders. Thus, acetazolamide inhibits both $I_{Cl^-}^{ms}$ and I_{sc} in the presence of Na^+ in ouabain-treated bladders (Table VI), as well as in the absence of ambient Na^+ (Table I).

Effect of acetazolamide on Mg^{2+}-dependent and $(Na^+ + K^+)$-stimulated ATPase

ATP splitting activity has been associated with transport phenomena in many tissues. Two moieties, a Mg^{2+}-dependent ATPase and a $(Na^+ + K^+)$-stimulated ATPase activity have been found in microsomal fractions of mucosal cells in the turtle bladder[7].

Mean values of three experiments presented in Table VI showed no change in the $(Na^+ + K^+)$-stimulated ATPase activity when acetazolamide was added to the incubating mixture. This was to be expected from previous data showing that the

active transport mechanisms of Cl^- and HCO_3^- are independent from the active transport mechanism of Na^+ and from the fact that acetazolamide was able to inhibit the active transport of Cl^- and HCO_3^- in the presence of ouabain, an inhibitor of the $(Na^+ + K^+)$-stimulated ATPase activity. An interesting, although negative, finding was that the Mg^{2+}-dependent ATPase activity was not modified by the presence of acetazolamide, in the incubating mixture. Such negative findings fail to provide a mean for relating the ATPase activities with the active transport of Cl^- and HCO_3^-.

TABLE VII

EFFECT OF ACETAZOLAMIDE ON ATPase ACTIVITY IN THE TURTLE BLADDER

Average values of three experiments in which acetazolamide 0.1 mM was added to tubes with and without ouabain. Incubation mixture contained, in final mM concentration, Tris–ATP, 2.0; EDTA, 0.2; imidazole·HCl, 40; histidine·HCl, 40; NaCl, 96; KCl, 24; $MgCl_2$, 3; final pH, 7.3.

Inhibitor	*Specific activity (μmoles P_i per mg protein per h)*		
	(A) $Mg^{2+} + Na^+ + K^+$ + *ouabain*	*(B)* $Mg^{2+} + Na^+ + K^+$	*(B) − (A)*
None	23.6	41.6	18.0
Acetazolamide (0.1 mM)	24.5	44.7	20.2

DISCUSSION

Inhibitors of carbonic anhydrase have profound influence on the rate of transport of ions in several tissues. Consequently, carbonic anhydrase has been implicated in the mechanism of these transport processes.

The fish gill[19] actively transports Na^+ and Cl^-. A definite relationship between carbonic anhydrase and the transport process has been suggested. This enzyme apparently catalyzes the formation of HCO_3^- and NH_4^+, both necessary for exchange with Cl^- and Na^+, respectively, across the external surface of the gill.

REHM *et al.*[20], using the carbonic anhydrase inhibitor acetazolamide, were able to inhibit significantly high secretion rate of H^+ in canine stomach with intact blood supply. The same drug failed to inhibit low secretion rate of H^+, but significantly inhibited the active transport of Cl^- in the isolated frog stomach[18,20,22]. The potency of several sulfonamides as inhibitors toward the active transport of Cl^- was found to be proportional to their relative activity as inhibitors of carbonic anhydrase[18,22].

These observations suggested that carbonic anhydrase was in some way related to the active transport of Cl^-. Two other findings seem to cast doubt upon the existence of such a relationship. (1) The concentrations of inhibitors required for the effect on transport are 100 times greater than those required to inhibit the isolated carbonic anhydrase activity. This difference could not be related to a delayed diffusion of the drug into the cells[22]. (2) Carbonic anhydrase inhibitors affect Cl^- transport in a tissue devoid of carbonic anhydrase, namely the frog cornea[23].

The turtle bladder is known to be a potent acidifier[9,11]. Furthermore, STEINMETZ[6] found that acetazolamide inhibits 80 % of the acidification process in bladders bathed

in HCO_3^--free solutions. At the time, this inhibitory action was related to a possible action of acetazolamide on carbonic anhydrase. Subsequently, MAREN[12] has reported that the homogenates of the turtle bladder do not possess carbonic anhydrase activity.

Data presented in this paper illustrate three important points. (1) Acetazolamide inhibits the net flux of Cl^- and the short-circuiting current associated with the presence of HCO_3^- in the mucosal fluid. (2) The short-circuiting current associated with HCO_3^- did not vary in the absence or presence of Cl^-. Conversely, it has been shown previously[8], that the net flux of Cl^- did not vary in the absence or presence of HCO_3^-.

Since the flow of bicarbonate and the flow of Cl^- do not seem to change appreciably when the ions are flowing singularly or simultaneously, it can be postulated that each ion travels through an independent parallel pathway.

Since both flows are due to active transport processes and seem to be uncoupled from each other and from the flow of other ions, and since the bladders studied were bathed on both mucosal and serosal surfaces by identical Ringer solutions, the transbladder PD can be represented using the thermodynamic treatment proposed by KEDEM[24], in the following way:

$$PD = \frac{(R_{HCO_3^-} R_{Cl^-})}{R_{HCO_3^-} + R_{Cl^-}} \left(\frac{R_{HCO_3^-,\gamma}}{R_{HCO_3^-}} J_\gamma^{HCO_3^-} + \frac{R_{Cl^-,\gamma}}{R_{Cl^-}} J_\gamma^{Cl^-} \right)$$

The term $(R_{HCO_3^-} R_{Cl^-})/(R_{HCO_3^-} + R_{Cl^-})$ represents the combined resistance of the bladder to both J^{Cl^-} and $J^{HCO_3^-}$ flowing through independent paths; $J_\gamma^{Cl^-}$ and $J_\gamma^{HCO_3^-}$ represent the flows of the chemical reactions associated with the Cl^- and HCO_3^- transport processes respectively and $R_{Cl^-,\gamma}$ and $R_{HCO_3^-,\gamma}$ are the coupling coefficients between the ion and the chemical flows.

Since both flows are in the same direction, the equation predicts that the PD should be related to the sum of their rates. This prediction is in accordance with the third important finding of this report and of the previous one[8]; namely that the transbladder potential is a function of the presence of Cl^- and HCO_3^- in the mucosal fluid. For example, values of PD decreased to about half their original values when either Cl^- or HCO_3^- were removed and replaced by the impermeant anion, SO_4^{2-}.

The action of acetazolamide could be due to an inhibitory effect on a driving reaction common to both Cl^- and HCO_3^- transport processes. This suggests that there is a common link for the two anions at some point in the transport sequence. On the other hand, the data presented suggests that there are two separate and independent transport pathways for Cl^- and HCO_3^-. These two possibilities can be reconciled if the reaction inhibited is not rate limiting when each ion is flowing alone, or when both ions are flowing simultaneously under the usual conditions *in vitro*; but becomes rate limiting in the presence of acetazolamide. Such a chemical process could be related to the functions of the pump carrier itself or to the energy coupling process. An attempt has been made in this report to relate the inhibitory effect of acetazolamide to the known ATP-splitting activities of the preparation studied. Acetazolamide inhibits both the active transport of Cl^- and the active transport of HCO_3^- in ouabain-treated bladders and fails to decrease the $(Na^+ + K^+)$-stimulated and the Mg^{2+}-dependent ATPase activities of the tissue. These findings suggest that acetazolamide inhibits a driving reaction which is not related to the microsomal ATPase activities.

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