J. Membrane Biol. 47, 27–37 (1979)

Pathways for Bicarbonate Transfer across the Serosal Membrane of Turtle Urinary Bladder: Studies with a Disulfonic Stilbene

Russell F. Husted, Loren H. Cohen, and Philip R. Steinmetz

Department of Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242

Received 21 July 1978; revised 23 October 1978; revised again 27 December 1978

Summary. Bicarbonate is transferred across the serosal (*S*) membrane of the epithelial cells of the turtle bladder in two directions. Cellular HCO_3^- generated behind the H^+ pump moves across this membrane into the serosal solution. This efflux of HCO_3^- is inhibited by SITS (4-isothiocyano-4′-acetamido-2,2′-disulfonic stilbene). When HCO_3^- is added to the serosal solution it is transported across the epithelium in exchange for absorbed Cl^-. This secretory HCO_3^- flow traverses the serosal cell membrane in the opposite direction. In this study the effects of serosal addition of 5×10^{-4} M SITS on HCO_3^- secretion and Cl^- absorption were examined. The rate of H^+ secretion was brought to zero by an opposing pH gradient, and 20 mM HCO_3^- was added to *S*. HCO_3^- secretion, measured by pH stat titration, was equivalent to the increase in $M \rightarrow S$ Cl^- flux after HCO_3^- addition. Neither the $S \rightarrow M$ flux of HCO_3^- nor the $M \rightarrow S$ flux of Cl^- were affected by SITS. In the absence of electrochemical gradients, net Cl^- absorption was observed only in the presence of HCO_3^- in the media; under such conditions, unidirectional and net fluxes of Cl^- were not altered by serosal or mucosal SITS. H^+ secretion, however, measured simultaneously as the short-circuit current in ouabain-treated bladders decreased markedly after serosal SITS. The inhibition of the efflux of HCO_3^- in series with the H^+ pump and the failure of SITS to affect HCO_3^- secretion and Cl^- absorption suggest that the epithelium contains at least two types of transport systems for bicarbonate in the serosal membrane.

The isolated bladder of the fresh water turtle is capable of net secretion of both acid and alkali [8, 16]. Acidification of the mucosal solution can be demonstrated in the absence of exogenous bicarbonate and occurs by a mechanism of hydrogen ion secretion [12, 15, 16]. The hydroxyl ions dissociated behind the H^+ pump are buffered by CO_2 within the cell, and the bicarbonate so formed moves passively out of the cell into the serosal solution. When bicarbonate, however, is added to the serosal solution it is transported across the epithelium into the mucosal solution. This secretory flow of bicarbonate is coupled to an absorptive flow of chloride [8]. Bicarbonate, therefore, must move in both directions across the serosal membrane of the epithelium.

0022-2631/79/0047-0027/$02.20

The present study was undertaken to explore whether there is an interaction between the bicarbonate efflux across the serosal membrane and the chloride-bicarbonate exchange flows. SITS (4-isothiocyano-4′-acetamido-2,2′-disulfonicstilbene) has been shown to inhibit urinary acidification [2, 3]. This inhibition is due to interference with the efflux of bicarbonate across the serosal membrane [2]. We therefore examined the effects of this agent on the exchange flows of chloride and bicarbonate. SITS did not significantly affect the secretory bicarbonate flow or the chloride fluxes. These results indicate that the serosal membrane sites for the movement of bicarbonate in the anion exchange pathway are separate from the bicarbonate efflux sites in series with the H^+ pump.

Materials and Methods

Urinary bladders of fresh water turtles, *Pseudemys scripta,* were mounted on Lucite chambers with an exposed surface area of 8 cm^2. The bladders were maintained in the short-circuited state by voltage clamping except for brief periods when the open-circuit potential difference was measured.

The compositions of the Ringer's solutions are shown in Table 1. The control and HCO_3^- Ringer's solutions were used in the first group of experiments. The sodium bicarbonate of the HCO_3^- Ringer's was osmotically balanced by sodium sulfate in the control Ringer's. The two Ringer's solutions with 27 mM Cl^- were used in subsequent experiments to reduce passive chloride fluxes. The sodium chloride of the control Ringer's was replaced isonatrically by sodium sulfate to make the 27 mM Cl^- Ringer's, and sucrose was used to maintain osmolality.

In the first group of experiments, bladders were mounted with control Ringer's in both the mucosal (*M*) and serosal (*S*) solutions. Mucosal pH was lowered until mucosal acidification was abolished (*M*pH 4.63 ± 0.06) so that the rate of bicarbonate secretion could be measured in the absence of hydrogen secretion [10, 17]. Cl^{36} (New England Nuclear, Boston, Mass.) was added to the mucosal solution and one hour later the steady-state chloride flux was determined. With the mucosal pH held constant by a Radiometer pH stat assembly, the serosal solution was then exchanged for 20 mM HCO_3^- Ringer's. After a 1-hr equilibration period, the steady-state bicarbonate and chloride flux rates were determined. SITS (Polysciences, Inc., Warrington, Pa.) was added to either the mucosal or serosal solution to attain a concentration of 5×10^{-4} M. After one hour the rates of chloride and bicarbonate flux were again determined. Bicarbonate flux was measured by pH stat titration and chloride by the flux of Cl^{36}.

For the second group of experiments, two hemibladders from the same whole bladder were mounted in 27 mM chloride Ringer's. One hemibladder of each pair was used to determine $S \rightarrow M$ chloride flux, and its mate was used for $M \rightarrow S$ flux determination. Chloride flux was determined during two one-half hour periods beginning one hour after Cl^{36} had been added to the appropriate bathing solution. The mucosal and serosal bathing solutions were then exchanged for 27 mM chloride $+HCO_3^-$ Ringer's and the Cl^{36} was replaced. Two half-hour flux measurements were obtained after a 1-hr equilibration period (control period). SITS was then added to the serosal solution of both halves of the experi-

Table 1. Composition of Ringer's solutions

	Control Ringer's	HCO_3^- Ringer's	27 mM Cl^- Ringer's	27 mM Cl^- + HCO_3^- Ringer's
	(mmol/liter)			
Na^+	123.4	116.8	123.4	116.8
K^+	3.5	3.5	3.5	3.5
Ca^{++}	1.8	1.8	1.8	1.8
Cl^-	101.5	101.5	27.1	27.1
$HPO_4^=$	1.2	1.2	1.2	1.2
$SO_4^=$	13.3	–	50.5	37.2
HCO_3^-	–	20	–	20
Sucrose	–	–	40	40
Gas Phase	CO_2-free Air	CO_2-free Air	CO_2-free Air	5% CO_2 in air
pH	7.1	8.4	7.1	7.25

mental bladders to attain a 5×10^{-4}M concentration. Chloride flux was measured during two additional half hour periods beginning 1 hr after SITS addition (experimental period).

Similar experiments were completed in two additional groups of bladders in which net sodium transport was abolished by ouabain. In these groups of bladders, HCO_3^- Ringer's with 27 mM chloride was present on both sides of the bladder throughout the experiment. Ouabain (5×10^{-4}M) was added to the serosal solution, and the reversed short-circuit current was measured to estimate the rate of urinary acidification. In one group 5×10^{-4}M SITS was added to the serosal solution, and in the other group SITS was added to the mucosal solution.

Analysis of Data

All data are reported as the mean ±SEM per 8 cm^2 bladder surface. The statistical significance of the data was determined by *t* test, analysis of variance, or by paired *t* test when appropriate [13].

Results

1. Effect of SITS on Simultaneously Measured, Oppositely Directed HCO_3^- and Cl^- Fluxes

The effects of serosal addition of SITS on the unidirectional bicarbonate and chloride fluxes are shown in Table 2. Bicarbonate was added only to the serosal (*S*) solution, and the transport into the mucosal (*M*) solution was measured by pH stat titration. The chloride flux from *M* to *S* was measured simultaneously. None of the bicarbonate fluxes

Table 2. Effect of serosal SITS on $S \rightarrow M$ bicarbonate ($J_{HCO_3^-}$) and $M \rightarrow S$ chloride flux (J_{Cl})

	HCO_3^--free	Control 20 mM HCO_3^-	Experimental 20 mM HCO_3^-
		(μmol/hr)	
Control ($n=8$)			
$J_{HCO_3^-}$	<0.02	1.16 ± 0.22	0.93 ± 0.18
J_{Cl^-}	2.20 ± 0.55	3.27 ± 0.83	3.10 ± 0.72
SITS ($n=7$)			
$J_{HCO_3^-}$	<0.02	0.99 ± 0.10	1.05 ± 0.13
J_{Cl^-}	2.52 ± 0.73	3.48 ± 0.85	3.47 ± 0.94

SITS was added to the serosal solution in a final concentration of 5×10^{-4}M.

were significantly different by analysis of variance. Likewise, all values of chloride flux during the control and experimental periods were statistically equivalent.

A bicarbonate-dependent chloride flux can be defined for these experiments as the difference between the M to S chloride flux in the presence and absence of serosal bicarbonate (second or third column minus first column of Table 2). During the control period the bicarbonate-dependent chloride flux was 1.07 μmol/hr in the control bladders and 0.94 μmol/hr in the bladders subsequently exposed to SITS. These values of chloride flux were not significantly different from the simultaneously measured values during the experimental period in both control and SITS-treated bladders.

Mucosal addition of 5×10^{-4}M SITS had no effect on the S to M bicarbonate flux in 5 experiments. The rate before SITS was 1.20 ± 0.17 μmol/hr. One hour after SITS the rate was 1.20 ± 0.16 μmol/hr. Previous studies have shown that mucosal addition of SITS had no effect on H^+ secretion [2] or on the short-circuit current in sodium-free media [3].

2. Effect of Serosal SITS on Unidirectional Cl^- Fluxes in Symmetrical Solutions

Unidirectional Cl^- fluxes were measured in paired halves of the same bladders, first in the HCO_3-free solutions and thereafter in the presence of 20 mM HCO_3^- on both surfaces of the bladder. To reduce passive Cl^- fluxes the Cl^- concentrations of the Ringer's solutions were reduced

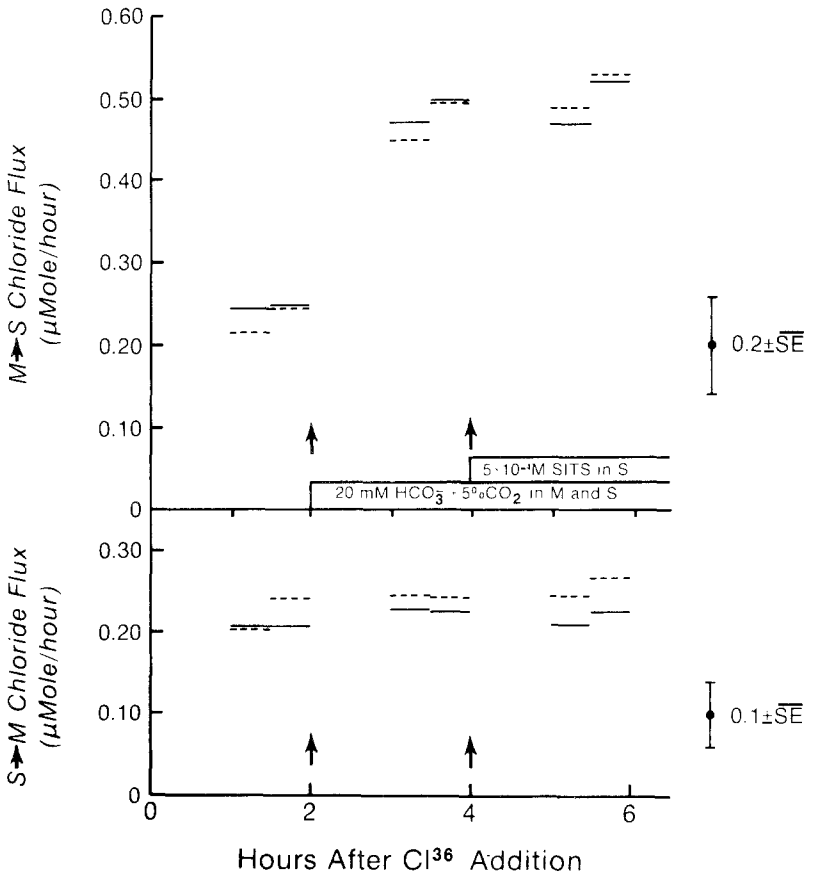

Fig. 1. Stimulation of absorptive chloride flux by bicarbonate addition and failure of SITS to affect unidirectional or net chloride fluxes. Solid lines represent control bladders ($n=5$); dashed lines represent SITS-treated bladders ($n=5$). An average SE is indicated on the right side of the figure

to 27.1 mM (*see* Table 1). In contrast to the previous experiments in which mucosal pH was 4.63 ± 0.06 (i.e., at the level of zero net H^+ secretion), there were no significant transepithelial pH or HCO_3^- gradients in this group of experiments (pH 7.25).

The effects of 20 mM HCO_3^- and serosal SITS on $S \rightarrow M$ and $M \rightarrow S$ chloride flux are shown in Fig. 1. In the absence of bicarbonate, $M \rightarrow S$ and $S \rightarrow M$ chloride fluxes were not significantly different. The addition of 20 mM HCO_3^- to the solutions bathing both sides of the bladder did not cause a significant change in $S \rightarrow M$ chloride flux, but increased the $M \rightarrow S$ chloride flux by more than 0.2 μmol/hr in accord with previous studies [8]. The serosal addition of 5×10^{-4}M SITS had no significant

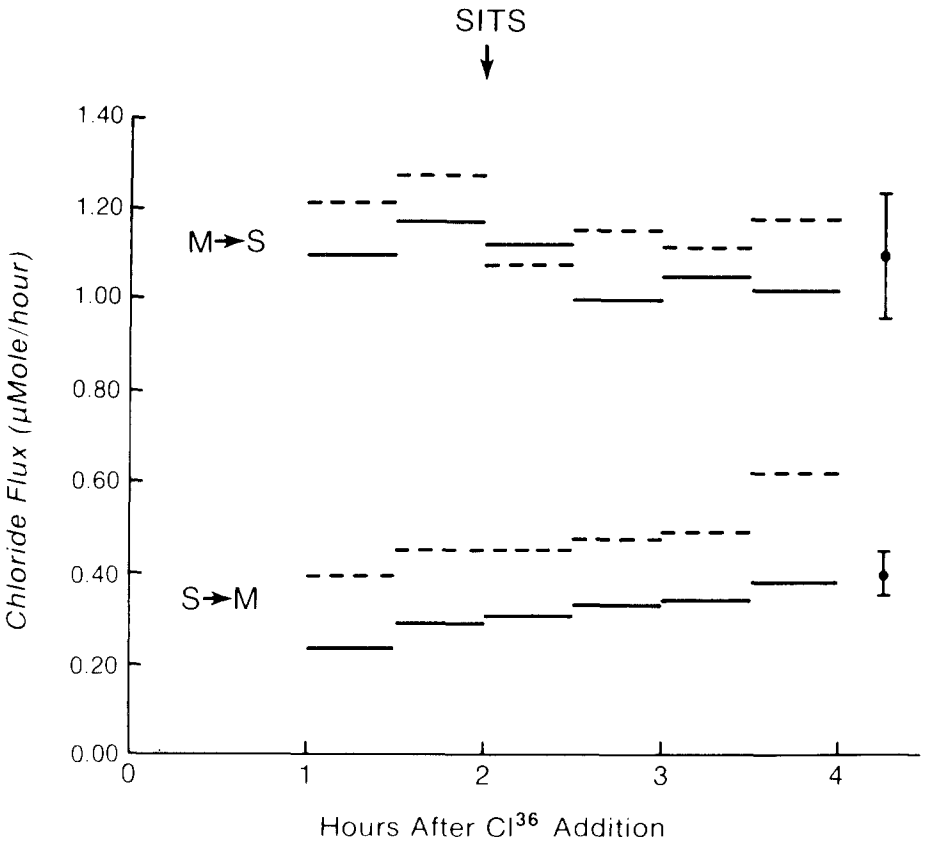

Fig. 2. Failure of serosal SITS (5×10^{-4}M) to affect chloride fluxes in the presence of 20 mM HCO_3^- and 5×10^{-4}M ouabain. Solid lines represent 4 control bladders, dashed lines represent experiments in which SITS was added to the serosal solution 2 hr after Cl^{36} addition ($M\rightarrow S$ flux in 6 hemibladders and $S\rightarrow M$ in 5 hemibladders). $S\rightarrow M$ flux increased ($P<0.02$) with time in both control and SITS-treated bladders. The rate of increase was similar ($P<0.2$) in both groups

effect on either the $S\rightarrow M$ or the $M\rightarrow S$ chloride fluxes. As shown on the right of Fig. 1, the fluxes in control and SITS-exposed halves were the same.

3. Effect of SITS on Cl^- Fluxes and on H^+ Secretion in Ouabain-Treated Bladders

The effects of SITS were further examined in bladders in which net Na^+ transport was abolished by 5×10^{-4}M ouabain. In such bladders bathed by symmetrical solutions, the rate of H^+ secretion approximates

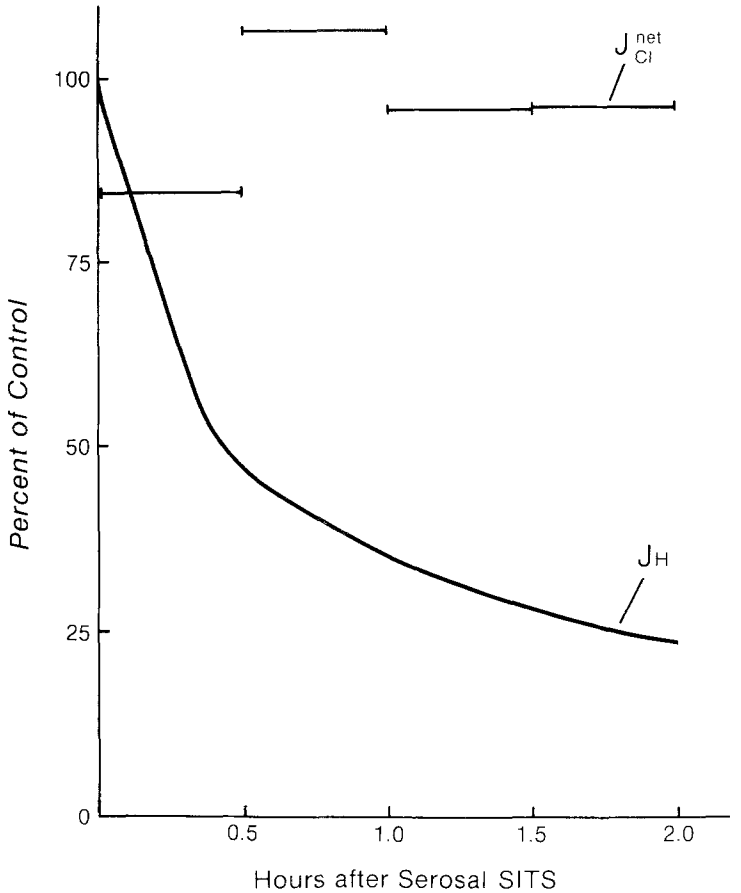

Fig. 3. Selective inhibition of H^+ secretion (J_H) by 5×10^{-4}M SITS. Net chloride flux (J_{Cl}^{net}) measured simultaneously was unaffected. J_{Cl}^{net} was derived from the unidirectional fluxes shown in Fig. 2. Control J_H in this group of ouabain-treated bladders was 70 ± 16 μA

the short-circuit current [11, 16]. Furthermore, previous studies [8, 14] have shown that the rates of HCO_3^- secretion and net Cl^- absorption are not affected by ouabain.

The effects of serosal SITS on the unidirectional $S \rightarrow M$ and $M \rightarrow S$ chloride fluxes are shown in Fig. 2. Symmetrical HCO_3^- Ringer's solutions were used as in the experiments above. The $M \rightarrow S$ fluxes were greater than the $S \rightarrow M$ fluxes, thus demonstrating net Cl^- absorption. The $S \rightarrow M$ fluxes increased with time in both the control and SITS-treated tissues. The addition of SITS to the serosal solution (dashed lines) had no significant effect on either flux compared to control values (solid lines). In Fig. 3 the net Cl^- fluxes from these experiments are

Table 3. Effect of mucosal SITS (5×10^{-4} M) on unidirectional chloride fluxes

	Control period	Experimental period
	$M \rightarrow S$ flux (μmol/hr)	
Control ($n=5$)	1.02 ± 0.02	1.00 ± 0.02
SITS ($n=5$)	1.22 ± 0.24	1.08 ± 0.24
	$S \rightarrow M$ flux (μmol/hr)	
Control ($n=5$)	0.33 ± 0.04	0.41 ± 0.04
SITS ($n=5$)	0.26 ± 0.04	0.36 ± 0.06

indicated by bars (J_{Cl}^{net}). J_{Cl}^{net} was unaffected by serosal SITS, whereas the short-circuit current (J_H) was markedly inhibited (solid curve). The inhibition of J_H reached 76% by 2 hr, a degree of inhibition comparable to that reported previously [2]. Ehrenspeck and Brodsky [3] have reported that serosal, but not mucosal, SITS inhibits the short-circuit current remaining in the absence of sodium transport.

In addition mucosal SITS did not significantly alter either $M \rightarrow S$ or $S \rightarrow M$ chloride fluxes measured between symmetrical HCO_3-containing solutions. The results of these experiments are shown in Table 3. As in the previous group of experiments, $S \rightarrow M$ chloride flux increased ($P<0.05$) with time but this increase was not altered by SITS. The changes in $M \rightarrow S$ chloride flux were not statistically significant in either control bladders or bladders exposed to mucosal SITS.

Discussion

The main results of this investigation demonstrate that the addition of SITS to the serosal side of the turtle bladder epithelium causes a highly selective inhibition of anion transport. As shown previously by Cohen, Mueller and Steinmetz [2], the efflux of HCO_3^-, which is generated in series with the H^+ pump, is markedly inhibited by SITS. In contrast, the present study shows that the coupled flows of Cl^- and HCO_3^- are SITS-insensitive. Although it is not known whether the two anion flows are coupled at the serosal or the mucosal membrane, it is clear that HCO_3^- entry from the serosal solution into the cell and Cl^- exit from the cell to the serosal solution are unaffected by the disulfonic stilbene.

The disulfonic stilbenes also inhibit the various anion transport systems of other tissues to differing extents. In the red blood cell, the

exchange flows of Cl^- and $SO_4^=$ are inhibited completely by the disulfonic stilbenes [1, 7] but the net efflux of Cl^- from the red cell is only partially inhibited [6]. In Ehrlich ascites tumor cells $SO_4^=$ movement is inhibited by SITS while Cl^- transport is SITS-insensitive [19].

The results of these experiments also confirm previous studies [8] indicating that net Cl^- absorption is dependent on the presence of serosal HCO_3^-. As in the study by Leslie, Schwartz and Steinmetz [8], the rates of HCO_3^- secretion and Cl^- absorption were comparable when HCO_3^- secretion was measured by pH stat titration after H^+ secretion was stopped by an opposing pH gradient. Net Cl^- absorption was also observed in the presence of symmetrical HCO_3^- solutions. The magnitude of net Cl^- absorption was somewhat greater in the ouabain-treated bladders which were studied in the fall (Figs. 2 and 3) than in the experiments which were carried out in the winter (Fig. 1). Seasonal variability in Cl^- absorption was also observed by Gonzalez, Shamoo and Brodsky [5]. The results of the present study are entirely consistent with the existence of an anion exchange transport system which depends on metabolic energy [10] and which secretes HCO_3^- in exchange for absorbed Cl^- [8].

Inhibition of H^+ secretion by the addition of SITS to the basolateral surface of epithelial cells has been observed not only in turtle urinary bladder, but also in rat proximal tubule [4, 18]. The inhibitory effect is comparable to that of acetazolamide and other carbonic anhydrase inhibitors in proximal tubules, but in turtle bladder SITS is a more potent inhibitor of H^+ secretion than acetazolamide [2]. Furthermore, the action of SITS is more selective than that of acetazolamide, since acetazolamide inhibits H^+ secretion as well as Cl-HCO_3 exchange transport in this reptilian tissue.

The selectivity of the SITS inhibition of anion transport across the serosal cell membrane of the turtle bladder suggests that the exchange flows of Cl^- and HCO_3^- occur at transport sites which are separate from the sites involved in the efflux of HCO_3^- in series with the H^+ pump. Although the cellular mechanisms responsible for this selectivity are not known, two possibilities may be considered. First of all, there could be different transport sites at the serosal cell membrane for the exit and entry of HCO_3^-, but a common transport pool for HCO_3^-. This possibility would be strengthened if one could demonstrate some interaction between the two HCO_3^- flows as a function of changes in the common pool. Our studies failed to show an increase in the rate of Cl-dependent HCO_3^- secretion after inhibition of the HCO_3^- exit step.

The possibility of a common HCO_3^- pool cannot be entirely excluded, however, since the cellular HCO_3^- concentration prior to SITS may have been close to the level at which the transport system becomes saturated.

A second interpretation of the SITS selectivity would be that the transport pathway for acidification involves a compartment for HCO_3^- disposition into *S* that is functionally separate from the anion exchange pathway for Cl^- and HCO_3^-. This interpretation appears to be the most attractive at the present time. Scanning electron microscopy [9] has revealed a marked alteration in the surface appearance of a cell population characterized by prominent microplicae 2 hr after SITS addition while the remainder of the cells appear to be unchanged. At that time H^+ secretion is markedly inhibited while Cl-HCO_3 exchange is unaltered. Whether or not these observations reflect that different epithelial cell types are involved in the two transport systems for bicarbonate remains to be explored.

This research was supported by NIH grants AM 17568 and HL 14388. R.F. Husted was the recipient of a postdoctoral fellowship under grant HL 07121.

This work was presented in part before the Fall Meeting of the American Physiological Society in 1977 in Hollywood, Florida.

References

1. Cabantchik, Z.I., Rothstein, A. 1972. The nature of the membrane sites controlling anion permeability of human red blood cells as determined by studies with disulfonic stilbene derivatives. *J. Membrane Biol.* **10**:311
2. Cohen, L.H., Mueller, A., Steinmetz, P.R. 1978. Inhibition of the bicarbonate exit step in urinary acidification by a disulfonic stilbene. *J. Clin. Invest.* **61**:981
3. Ehrenspeck, G., Brodsky, W.A. 1976. Effects of 4-acetamido-4′-isothiocyano-2,2′-disulfonic stilbene on ion transport in turtle bladders. *Biochim. Biophys. Acta* **419**:555
4. Fromter, E. 1977. Mechanism of H^+/HCO_3^- transport in rat kidney proximal tubule and mode of action of carbonic anhydrase inhibitors. *Proc. Int. Physiol. Sci.* **12**:204
5. Gonzalez, C.F., Shamoo, Y.E., Brodsky, W.A. 1967. Electrical nature of active chloride transport across short-circuited turtle bladders. *Am. J. Physiol.* **212**:641
6. Knauf, P.A., Fuhrmann, G.F., Rothstein, S., Rothstein, A. 1977. The relationship between anion exchange and net anion flow across the human red blood cell membrane. *J. Gen. Physiol.* **69**:363
7. Knauf, P.A., Rothstein, A. 1971. Chemical modification of membranes. I. Effects of sulfhydryl and amino reactive reagents on anion and cation permeability of the human red cell. *J. Gen. Physiol.* **58**:190
8. Leslie, B.R., Schwartz, J.H., Steinmetz, P.R. 1973. Coupling between Cl^- absorption and HCO_3^- secretion in turtle urinary bladder. *Am. J. Physiol.* **225**:610
9. Mueller, A.L., Kessel, R.G., Husted, R.F., Cohen, L.H., Steinmetz, P.R. 1978. Effects of a disulfonic stilbene (SITS) on surface morphology and urinary acidification in turtle bladder. *Fed. Proc.* **37(3)**:511

10. Oliver, J.A., Himmelstein, S., Steinmetz, P.R. 1975. Energy dependence of urinary bicarbonate secretion in turtle bladder. *J. Clin. Invest.* **55**:1003
11. Schwartz, J.H. 1976. H^+ current response to CO_2 and carbonic anhydrase inhibition in turtle bladder. *Am. J. Physiol.* **231**:565
12. Schwartz, J.H., Finn, J.T., Vaughn, G., Steinmetz, P.R. 1974. Distribution of metabolic CO_2 and the transported ion species in acidification by turtle bladder. *Am. J. Physiol.* **226**:283
13. Snedecor, G.W., Cochran, W.G. 1967. Statistical Methods. (6^{th} Ed.) Iowa State University Press, Ames
14. Solinger, R.E., Gonzalez, C.F., Shamoo, Y.E., Wyssbrod, H.R., Brodsky, W.A. 1968. Effect of ouabain on ion transport mechanisms in the isolated turtle bladder. *Am. J. Physiol.* **215**:249
15. Steinmetz, P.R. 1967. Characteristics of hydrogen ion transport in urinary bladder of water turtle. *J. Clin. Invest.* **46**:1531
16. Steinmetz, P.R. 1974. Cellular mechanisms of urinary acidification. *Physiol. Rev.* **54**:890
17. Steinmetz, P.R., Lawson, L.R. 1971. Effect of luminal pH on ion permeability and flows of Na^+ and H^+ in turtle bladder. *Am. J. Physiol.* **220**:1573
18. Ullrich, K.J., Capasso, G., Rumrich, G., Papavassilou, F., Kloss, S. 1977. Coupling between proximal tubular transport processes: Studies with ouabain, SITS and HCO_3^--free solutions. *Pfluegers Arch.* **368**:245
19. Villereal, M.L., Levinson, C. 1976. Inhibition of sulfate transport in Ehrlich ascites tumor cells by 4-acetamido-4′-isothiocyano-stilbene-2,2′-disulfonic acid (SITS). *J. Cell. Physiol.* **89**:303