for discussion; C. K. Brain, W. D. Haacke, G. and M. Mills, and C. and E. LeRiche for assistance in Africa; and directors of Wildlife and National Parks (Botswana), National Parks Board (Republic of South Africa), and Nature Conservation (Cape Province) for permission to work in their respective areas. Research supported by the National Geographic Society, National Science Foundation (grant GB-8727), Los Angeles County Museum of Natural History, Miller Institute for Basic Research in Science, and the Museum of Vertebrate Zoology and Department of Zoology (University of California, Berkeley).

1 June 1976; revised 14 September 1976

Chloride Transport Across Isolated Opercular Epithelium of Killifish: A Membrane Rich in Chloride Cells

Abstract. *The opercular epithelium of* Fundulus heteroclitus *contains typical gill chloride-secreting cells at the high density of* 4×10^5 *cells per square centimeter. When isolated, mounted as a membrane, and short-circuited, it actively transports chloride ions from the blood side to the seawater side of the preparation. This preparation offers a useful approach to the study of osmoregulation in bony fishes.*

Extrarenal salt secretion is the key to the seawater teleost's ability to maintain an internal salt concentration hypoosmotic to its environment. To date, this osmoregulatory mechanism has been studied either in intact animals or in isolated, perfused gills (*1*). However, neither of these preparations meets all the criteria necessary for the application of the short-circuit current technique classically used in the study of ion transport across epithelia. The gill and the opercular epithelium have similar electrical and cytological characteristics, but the short-circuited opercular epithelium of the killifish (*Fundulus heteroclitus*) offers a better biophysical approach to the study of osmoregulation in teleosts. This tissue is a stratified epithelium that includes a small fraction of pavement, mucous, and nondifferentiated cells, and 50 to 70 percent chloride-secreting cells (*2–4*). The chloride-secreting cells extend from the basal lamina to the external aquatic environment and interrupt the stratification. Each cell has a prominent apical crypt, a rich population of mitochondria, and an extensive, branching tubular system continuous with the basal and lateral plasma membrane; these cells are identical in fine structure to the chloride-secreting cells of the gill (*3*).

The epithelium lining the pharyngeal cavity of the seawater-adapted killifish (*5*) was dissected free of the bony operculum and mounted as a flat sheet in a Lucite chamber with an aperture of 0.07 cm^2 (*6*). When bathed in the appropriate salt solution (*7*), the transepithelial potential difference initially increased within an hour to between 10 and 40 mv (mean ± standard error = 24.0 ± 1.6; $N = 32$; seawater side negative) and remained relatively constant for several hours. These potential difference values are in good agreement with those measurements made in vivo between the seawater environment and the plasma in several species of teleosts (*1*), and are somewhat higher than those observed in isolated, perfused flounder gills bathed on both sides with Ringer solution (*8*). The short-circuit current (I_{sc}) increased in a manner similar to the potential difference and reached steady state levels between 70 and 340 $\mu a/cm^2$ (mean = 190.1 ± 13.1; $N = 32$) where it remained constant for several hours. The transepithelial d-c resistance, taken as the ratio PD/I_{sc} (where PD is the potential difference), ranged from 70 to 330 ohm-cm^2 (mean = 138.5 ± 9.2, $N = 32$) and had the tendency to increase slightly over the course of several hours. Initial current-voltage relationship studies across this tissue showed a linear response indicating that this tissue behaved as an ohmic resistor and justified the use of this ratio as an indicator of the total transepithelial d-c resistance. The procedure for short-circuiting and the isotope flux measurements used here are described elsewhere (*9*).

As shown in Fig. 1a and Table 1, nitrogen-induced anoxia caused a significant ($P < .01$) reduction in the I_{sc} and potential difference, which was quickly reversed with the reintroduction of oxygen into the system. The substitution of chloride ion with the nonpenetrating methylsulfate anion in the solutions bathing both sides of the epithelium (Fig. 1b and Table 1) caused a rapid and significant ($P < .005$) decline in both the I_{sc} and potential difference to near zero values which were readily reversed with the reintroduction of chloride ions into the

Table 1. Chloride ion substitution and the effect of chloride transport inhibitors on the isolated opercular epithelium of *F. heteroclitus*. The data are expressed as means ± standard error. Numbers of experiments are in parentheses; R, resistance.

Experiment	Average I_{sc} ($\mu a/cm^2$)	Average PD (mv)	Average R (ohm-cm^2)	Average time to maximum effect (minutes)
Control (6)	234.4 ± 30.5	26.1 ± 4.0	122.7 ± 20.5	
Chloride free	8.8 ± 3.9	1.9 ± 0.6	58.5 ± 40.0	27
Percentage change	96.3	92.7	52.3	
Chloride rich	224.5 ± 27.8	23.5 ± 2.5	121.3 ± 28.9	5
Control (4)	236.6 ± 4.7	26.3 ± 2.5	110.7 ± 9.8	
Anoxia, N_2	39.6 ± 10.4	8.6 ± 1.7	269.2 ± 74.7	30
Percentage change	83.3	67.3	143.2	
Oxygenation	233.0 ± 14.7	30.8 ± 2.6	131.9 ± 5.3	11
Control (4)	186.4 ± 39.1	30.9 ± 4.0	186.3 ± 46.2	
Furosemide, $10^{-3}M$	22.9 ± 13.1	3.6 ± 1.2	173.3 ± 69.1	30
Percentage change	84.3	88.3	7.0	
Control (4)	139.7 ± 40.9	18.2 ± 4.1	141.6 ± 24.2	
Ouabain, $10^{-5}M$	15.9 ± 9.9	1.3 ± 0.8	79.4 ± 10.6	87
Percentage change	88.6	92.9	43.9	
Control (2)	115.4 ± 8.3	17.7 ± 6.5	150.2 ± 45.6	
Thiocyanate, $10^{-2}M$	60.0 ± 6.4	10.5 ± 3.9	169.1 ± 47.8	24
Percentage change	48.0	40.7	12.6	

Table 2. Fluxes of Cl^- across the isolated, short-circuited opercular epithelium of *F. heteroclitus*. The data are expressed as means ± standard error. Numbers of experiments are in parentheses. The average unidirectional $^{36}Cl^-$ fluxes across the paired preparations were significantly different from each other ($P < .01$) while the difference between the average I_{sc} for the paired preparations was not significant ($P > .90$).

Fluxes [$\mu eq\ (cm^2)^{-1}\ hour^{-1}$]			Net flux ($\mu a/cm^2$)	Average I_{sc} ($\mu a/cm^2$)	[HCO_3^-] (mM)
Outflux	Influx	Net flux			
8.325 ± 1.668 (6)	1.162 ± 0.132 (6)	7.163 ± 1.739	191.9 ± 46.6	183.8 ± 21.8	16
3.452 ± 0.131 (2)	1.929 ± 0.009 (2)	1.523 ± 0.140	40.8 ± 3.7	39.0 ± 4.8	4

bathing solutions. The stepwise titration of chloride ions into both solutions simultaneously resulted in a corresponding increase in both the I_{sc} and potential difference. The I_{sc} returned to near control values when the chloride concentration approached that of the original bathing solution and the potential difference increased linearly with increasing chloride concentrations with a Nernst slope of 28 mv per tenfold concentration change (*10*).

The $^{36}Cl^-$ fluxes were measured across paired pieces of opercular epithelium obtained from the same fish and mounted in matched chambers. The blood to seawater flux (outflux) was measured in one epithelium while the seawater to blood flux (influx) was measured in the companion epithelium. The preparations were kept short-circuited except for a few seconds every 30 minutes to record the potential difference and calculate the d-c resistance. The unidirectional chloride fluxes were determined from half-hour sampling periods and the net flux was calculated as the difference between the unidirectional fluxes across the paired preparations. The average results of six paired flux experiments are presented in Table 2. The average $^{36}Cl^-$ outflux was approximately seven times the $^{36}Cl^-$ influx, resulting in a net outflux which was 4.4 percent higher than the average I_{sc} for all these flux experiments. That the difference between the average net chloride current and the average I_{sc} was not statistically significant ($P > .90$) indicated that most of the I_{sc} could be attributed to the active transport of chloride ions from the blood to the seawater side of this epithelium. However, because I_{sc} is a measure of the net movement of charge across a tissue, these data do not exclude the possibility that other ions may be actively transported across this preparation. It is interesting that previous evidence obtained in vivo or in isolated gill preparations pointed toward chloride transport (*1, 8*), but only with this isolated, short-circuited opercular preparation is it possible to conclude on more solid grounds that a net active transport of this ion exists.

Chloride-bicarbonate interactions have been reported in a variety of transporting epithelia (*11*). Table 2 also presents the average unidirectional and net $^{36}Cl^-$ fluxes across the opercular epithelium bathed on both sides with low concentrations (4 m*M*) of bicarbonate. The average $^{36}Cl^-$ outflux was 41.5 percent of that in high concentrations (16 m*M*) of bicarbonate, while the average $^{36}Cl^-$ influx was 66.0 percent higher than that in high bicarbonate concentrations. The outflux was still greater than the influx but the net flux was reduced to 21.3 percent of that observed in high bicarbonate concentrations. However, in these flux studies there was no significant difference ($P > .70$) between the average I_{sc} and the net $^{36}Cl^-$ current for low concentrations of bicarbonate.

The stepwise addition of bicarbonate to the solutions bathing both sides of the opercular epithelium produced corresponding increases in the I_{sc} and potential difference. Small increases occurred in the *p*H of these solutions, but similar *p*H changes produced by the titration of sodium hydroxide into the bathing solutions produced no stimulatory effects on the I_{sc} or potential difference. Although the standard bathing solutions contained 16 m*M* bicarbonate, the electrical properties across this isolated preparation could be further stimulated by much higher concentrations of bicarbonate, as illustrated in Fig. 1c. The equivalency of the net $^{36}Cl^-$ transport and the I_{sc} in the presence of high and low concentrations of bicarbonate makes it unlikely that a neutral Cl^-/HCO_3^- transepithelial exchange occurred. The stimulatory action of bicarbonate on the I_{sc} suggests the possibility of an exchange mechanism between Cl^- and HCO_3^- operating at one of the membranes of the cell. However, Diamox (*12*), a known inhibitor of carbonic anhydrase, had no effect on the I_{sc} across this epithelium at a concentration of $10^{-2}M$ in both bathing solutions.

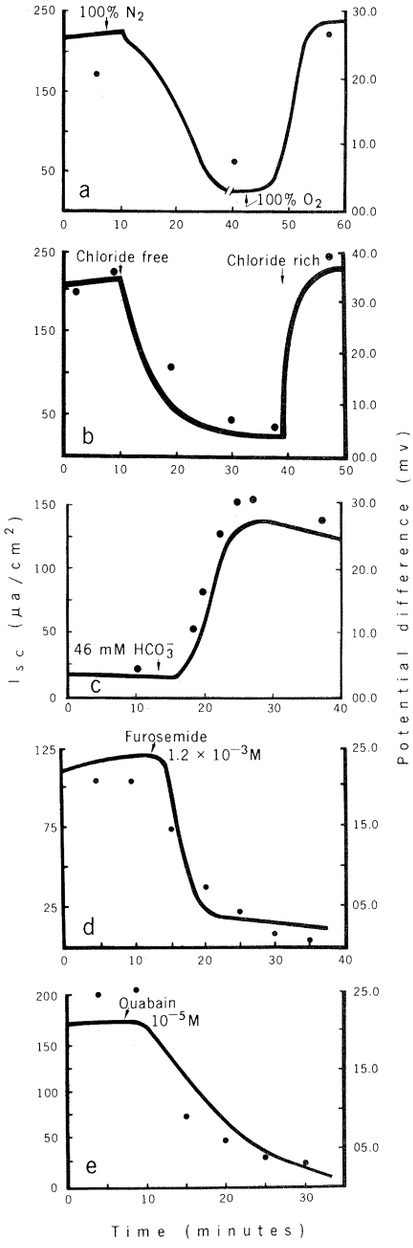

Fig. 1. Inhibition and stimulation of the electrical properties of the isolated opercular epithelium of *F. heteroclitus*. The figures represent individual experiments representative of the observed effect. The solid lines are continuous I_{sc} recordings and the circles are the *PD* values measured when the preparations were briefly open-circuited. (a) Effect of anoxia. (b) Effect of chloride-free Ringer solution. (c) Stimulatory effect of bicarbonate ion. (d) Inhibitory effect of furosemide. (e) Inhibitory effect of ouabain.

Known inhibitors of chloride transport were tested on these preparations and the results are listed in Table 1. Furosemide (*12*), which inhibits chloride transport in the kidney (*13*) and frog cornea (*14*), produced significant ($P < .01$) decreases in the I_{sc} and potential difference of 84.3 and 88.3 percent, respectively (Fig. 1d), which were not readily reversible. Ouabain (Fig. 1e), which is a specific inhibitor of $Na^+ + K^+$-stimulated adenosine triphosphatase (E.C. 3.6.1.3) and which also inhibits chloride transport in most epithelia engaged in this activity (*15*), produced an 88.6 percent ($P < .05$) and 92.9 percent ($P < .02$) decrease in the I_{sc} and potential difference, respectively. Thiocyanate, which inhibits its chloride secretion in intact fish and other chloride-transporting tissues (*16*), also had an inhibitory effect on the I_{sc} and potential difference across the opercular epithelium. At a concentration of $10^{-3}M$ on both sides of this tissue there was no effect of thiocyanate, whereas at $10^{-2}M$ the I_{sc} was inhibited by 48.0 percent ($P < .025$) and the potential difference by 40.7 percent ($P < .25$). The lack of a thiocyanate effect at $10^{-3}M$ and the magnitude of the potential difference decline at $10^{-2}M$ were strikingly similar to those effects of thiocyanate on the isolated, perfused flounder gill preparation (*8*). The equivalency of the I_{sc} and the net $^{36}Cl^-$ transport across this epithelium suggested that these drugs acted either directly on the active chloride ion transport pathway or indirectly, such as by

disrupting the normal Na^+ and K^+ distribution across the membrane, to inhibit the active chloride transport.

A number of features makes this tissue suitable for the study of transepithelial salt transport with the short-circuit current technique: the ready availability of the killifish; the ample surface area of the killifish opercular epithelium; the durability of the tissue in vitro; and the presence of large numbers of chloride-secreting cells in the tissue (*2*). The gill respiratory lamellae (*17*) present unsurmountable difficulties in studies of short-circuited gill filaments, and gills have a relatively small proportion of chloride-secreting cells (*4*). This opercular epithelium preparation may also be useful for studying active chloride transport across single cell membranes, in the same way that the giant neuron of *Aplysia* (*18*) is used in such studies.

Fundulus heteroclitus will adapt to a wide range of salinities, from fresh water to 200 percent seawater (*4*), and thus the opercular epithelium preparation may serve as a model for studies of the comparative aspects of ion transport mechanisms associated with euryhalinity. Extrarenal ion-secretory tissues, such as the salt glands (*19*), are also complex and cannot be studied as flat, short-circuited sheets. The killifish opercular epithelium may therefore be used as a general model for ion transport mechanisms in a broad range of osmoregulatory epithelia.

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2. Since their initial description in 1932 [A. Keys and E. N. Willmer, *J. Physiol. London* **76**, 368 (1932)], chloride-secreting cells of the gill epithelium have been assumed to be the cellular basis for osmoregulatory sodium and chloride secretion in seawater teleosts. Chloride-secreting cells were originally described in *F. heteroclitus* opercular epithelium by J. Burns and D. E. Copeland [*Biol. Bull. Woods Hole Mass.* **99**, 381 (1950)]. For an estimate of the relative percentage of the cell types, the chloride-secreting cell density, and a description of the fine structure of the opercular epithelium of *F. heteroclitus* see Karnaky and Kinter (*3*). For gill ultrastructure see C. W. Philpott [*Protoplasma* **60**, 7 (1965)], J. D. Newstead [*Z. Zellforsch.* **116**, 1 (1971)], and Karnaky *et al.* (*4*).
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4. ———, L. B. Kinter, C. E. Stirling, *J. Cell Biol.* **70**, 157 (1976).
5. Specimens of *F. heteroclitus* (6.0 to 14.0 g) were adapted for 2 to 5 weeks to 100 percent artificial seawater according to the technique described by Karnaky *et al.* (*4*). Each fish yielded two opercular epithelia, one from each operculum.
6. This small chamber has been described by J. A. Zadunaisky and K. J. Degnan [*Exp. Eye Res.* **23**, 191 (1976)] for small areas of ocular tissues. It consists of two hemidiscs between which the membrane is positioned across the aperture; the discs are then placed between the two halves of the chamber. The volume of each chamber half was 2.5 ml.
7. Modified Forster's medium [R. P. Forster, *Science* **108**, 65 (1948)]: NaCl, 135 m*M*; KCl, 2.5 m*M*; $CaCl_2$, 1.5 m*M*; $MgCl_2$, 1.0 m*M*; $NaHCO_3$, 16 m*M*; Trizma base, 20 m*M*. The *p*H was 8.25, and the solutions were gassed with 100 percent O_2 at 24° to 26°C.
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17. Gill filaments are the sites of osmoregulatory ion transport as well as of gas exchange, the latter being affected by the numerous respiratory leaflets protruding from the filaments. The application of the short-circuit current technique to this structurally complex gill has never been reported and should prove difficult with the techniques presently available.
18. J. M. Russell and A. M. Brown, *J. Gen. Physiol.* **60**, 499 (1972).
19. These salt glands are found in elasmobranchs, marine turtles and snakes, desert lizards, and birds.
20. This work was supported by NIH research grants AM 15973 (to W. B. Kinter) and EY 01340 (to J.A.Z.), and research fellowships GM 57244 (to K.J.K.) and EY 05059 (to K.J.D.).

13 July 1976; revised 8 September 1976

Diabetic Cataracts and Flavonoids

Abstract. *Oral administration of quercitrin, an inhibitor of aldose reductase, leads to a significant decrease in the accumulation of sorbitol in the lens of diabetic* Octodon degus. *The onset of cataract is effectively delayed when quercitrin is continuously administered. Thus in these diabetic animals, as in galactosemic rats, the use of an effective aldose reductase inhibitor impedes the course of cataract development. These observations support the hypothesis that in diabetes, as in galactosemia, aldose reductase plays a key role in initiating the formation of lens opacity.*

Aldose reductase has been implicated in the etiology of cataracts in diabetic and galactosemic animals (*1*, *2*). This enzyme catalyzes the reduction of glucose and galactose to their respective polyols, sorbitol and galactitol, by the reaction: Aldose + NADPH → Polyol + NADP [NADPH, reduced form of nicotinamide adenine dinucleotide phosphate (NADP)]. In diabetes and galactosemia these polyols attain a strikingly high level in the lens, leading to an osmotic overhydration of the tissue. This osmotic change initiates a series of physicochemical events that ultimately lead to the formation of mature cataracts. Thus, according to this theory, aldose reductase plays a key role in the cataractous process. The most convincing evidence in support of this hypothesis comes from in vivo experiments involving the inhibitors of aldose reductase. In galactosemic rats, systemic administration of an aldose reductase inhibitor effectively delays the onset of the cataractous process (*3*). In the diabetic situation, however, the support of this thesis comes mainly from the in vitro lens culture studies (*4*). Validation of the hypothesis from experiments with the intact diabetic animal has not been obtained because the rat develops a nuclear opacity after a long period of 3 to 4 months following the establishment of experimental diabetes. Under these circumstances it is difficult to establish the effectiveness of an in-

Table 1. Effect of quercitrin on the concentrations of sugars and polyols in the lenses of diabetic degus. Average blood glucose of animals was 465 ± 20 mg/100 ml; period of diabetes was 2 days. Experimental details have been described in the text. Number of analyses in each group was six. Results are expressed as micromoles per gram wet weight of the tissue ± standard deviation. Lenses were analyzed for the sugars and polyols by gas-liquid chromatographic technique described previously (*7*).

Animals	Glucose	Fructose	Sorbitol	Inositol
Treated	1.5 ± 0.1	4.1 ± 0.4	9.4 ± 1.1	1.5 ± 0.8
Nontreated	1.7 ± 0.1	8.4 ± 1.5	18.7 ± 1.9	1.1 ± 0.4