gland where SITS concentrations below one millimolar applied over extended periods of time did not interfere with the secretion of the perfused rectal gland.

Thus, in conclusion, this study as well as other experiments published in this bulletin (Hannafin J. et al., this bulletin) strongly suggest that the rectal gland contains a sodium-chloride (potassium) cotransport system that is involved in the active secretion of chloride by the gland. This work was supported by NIH Grants AM27441, AM29927 and the American Heart Association, Maine Affiliate #E-4.

ION FLUXES AND N-METHYLFUROSEMIDE BINDING IN SQUALUS ACANTHIAS RECTAL GLAND PLASMA MEMBRANE VESICLES

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INTRODUCTION--Recently a potassium dependence of Na, Cl cotransport has been described in avian erythrocyte (Palfrey et al., Am.J. Physiol. 238(7):C129-148, 1980), Ehrlich ascites tumor cells (Geck et al., Biochim. Biophys. Acta 600:432-447 1980), thick ascending limb of Henle's loop (Greger and Schlatter, Pfluegers Arch. 392:92-94, 1982) and in Madin-Darby canine kidney cell line (McRoberts et al., J. Biol. Chem. 257:2260-2266, 1982). Since the rectal gland is also supposed to contain a loop diuretic sensitive Na, Cl cotransport system it was of interest to examine the role of potassium in the rectal gland system. To this end three parameters for the presence of a Na, K, Cl cotransport system were investigated in rectal gland plasma membrane vesicles: potassium dependence of sodium flux, sodium dependence of potassium (rubidium) flux, and sodium and potassium dependence of the binding of a loop diuretic.

MATERIALS AND METHODS--Dogfish caught by hook and line in Frenchman Bay were maintained in live cars and used within two days to one week of capture. Isolation of plasma membrane vesicles was carried out at 0-4°C as described by(E.veloff et al., Pfluegers Arch. 378:87-92, 1978) with modifications. Protein was determined by the method of (Lowry et al., J. Biol. Chem. 193:265-275, 1951) using bovine serum albumin as standard. Na-K-ATPase activity was determined by the method of (Kinne et al., Pfluegers Arch. 329:191-206, 1971). ^{22}Na uptake and ^{86}Rb uptake were followed by a rapid filtration technique as described previously (Pfluegers Arch. 378:87-92, 1978). The binding of tritiated N-methylfurosemide was measured by the following technique. The reaction was initiated by the addition of 20 µl membranes to 65 µl incubation medium. After a 20 min, a 20 µl sample was removed and pipetted directly onto a mixed cellulose ester filter (Millipore HAWP, pore size 0.45 u) in 15-20 discrete drops kept under suction. The filter was then washed with 3.5 ml ice cold stop solution; washing took less than 2 seconds. Experiments were performed (in duplicate for transport and in triplicate for binding) at 15°C. Composition of the incubation media is given in the figure and table legends. For evaluation of the amount of radioactivity taken up by or bound to the vesicles the amount of radioactivity present on the filters was corrected for the amount of radioactivity present on the filter in the absence of membrane.

RESULTS--As shown in Table 1 KCl-gradient dependent ^{22}Na uptake decreased 53% when the K-concentration was decreased from 164 to 5 mM by choline replacement. ^{22}Na-uptake in the presence of a RbCl-gradient was slightly higher than the KCl-gradient dependent uptake, indicating that Rb is an appropriate replacement for K. In addition, 0.8×10^{-3} M N-methylfurosemide inhibited KCl-gradient dependent ^{22}Na-uptake after 15 seconds by 35% (data not shown).

Rubidium tracer exchange in the vesicles was found to be stimulated by the presence of chloride and sodium but inhibited by bumetanide. The latter inhibition was observed only in the presence of sodium and chloride (see Table 2).

The binding of [^{3}H] N-methylfurosemide to the membranes could be resolved into at least two components. One component demonstrates saturation kinetics (see Figure 1), and is thought to represent high affinity binding while the

Table 1.--Effect of K on Na Fluxes in Rectal Gland Plasma Membrane Vesicles

	^{22}Na Uptake (% 170 mM KCl Equilibrium)		
	15 sec	1 min	120 min
5 mM KCl (n = 3)	10.5 ± 1.4	19.3 ± 2.5	113.7 ± 4.4
10 mM KCL (n = 3)	11.5 ± 2.0	23.2 ± 1.4	103.0 ± 5.8
50 mM KCl (n = 3)	15.1 ± 1.6	34.2 ± 3.5	91.4 ± 1.5
170 mM KCl (n = 3)	22.5 ± 2.9	55.2 ± 7.0	100
170 mM RbCl (n = 2)	32.7 ± 2.9	79.8 ± 8.5	101.6 ± 6.4

^{22}Na uptake into plasma membrane vesicles. Vesicles were incubated in 200 mM mannitol, 20 mM Tris-HEPES, 1.2 mM Mg $(NO_3)_2$, 0.5 mM ^{22}Na NO_3, and 200 mM salt (KCl, KCl + Choline Cl, or RbCl). Mean values and standard error of the mean are expressed as % KCl equilibrium in the presence of 170 mM KCl.

Table 2.--^{86}Rubidium Tracer Exchange in Rectal Gland Plasma Membrane Vesicles

	k
NaCl	0.37 ± 0.04
NaCl + bumetanide*	0.16 ± 0.03
Choline Cl*	0.25 ± 0.03
Choline Cl + bumetanide*	0.20 ± 0.03
$NaNO_3$*	0.16 ± 0.01
$NaNO_3$ + bumetanide*	0.13 ± 0.02

*Significantly different ($p < 0.05$) from NaCl value. The vesicles were first suspended in 200 mM mannitol, 20 mM Tris-HEPES, 1.2 mM $Mg(NO_3)_2$, pH 7.6 then preincubated for two hours in media containing 200 mM mannitol, 0.5 mM $RbNO_3$, 20 mM Tris-HEPES, 1.2 mM $Mg(NO_3)_2$, pH 7.6 and 200 mM NaCl, Choline Cl, or $NaNO_3$. In uptake studies after this point, no statistically significant difference in the content could be detected in the various incubation media. Incubation media contained 200 mM mannitol, 0.5 mM $^{86}RbNO_3$, 20 mM Tris-HEPES, 1.2 mM $Mg(NO_3)_2$, 200 mM salt, pH 7.6. k for Rb exchange was derived by plotting $\ln (c_{\infty} - c_t/c_{\infty})$ versus time. Linear regression analysis was performed and k represents the calculated slope of the line. Mean values ± SEM derived from 3 experiments.

other is directly proportional to the concentration of N-methylfurosemide in the incubation medium and is thought to represent partition of the ligand into the membrane and or uptake into the intravesicular space. Scatchard analysis yields a K_D of 3.5 ± 1.2 x 10^{-6} M with a number of binding sites n equal to 104 ± 21 pmoles/mg protein. The binding of N-methylfurosemide is affected by the ionic composition of the incubation medium as shown in Table 3. In Na free or low K media a 51% decrease in N-methylfurosemide binding is observed, while the addition of 400 mM Cl reduces binding by 53%. In contrast, no effect was seen with 400 mM gluconate. In addition, other "loop diuretics" can be shown to compete with the binding of N-methylfurosemide as shown in Table 3 where 10^{-3} M bumetanide leads to a 56% decrease and 10^{-3} M furosemide to 75% decrease in binding.

DISCUSSION--Three properties of rectal gland plasma membranes have been studied; sodium uptake, rubidium uptake and binding of the diuretic N-methylfurosemide. In Figure 2 a scheme is presented that summarizes the results

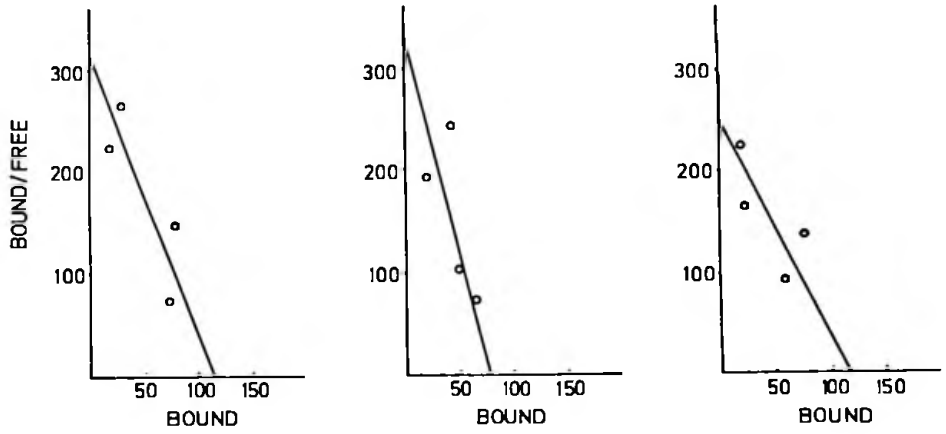

Figure 1.--Binding of N-methylfurosemide to plasma membrane vesicles isolated from dogfish rectal gland. The results of three experiments are shown after subtraction of a linear nonsaturable component. b = amount of N-methylfurosemide bound to the membranes in pmol/mg protein, f = free concentration of N-methylfurosemide in moles/l. The binding experiments were performed in 100 mM mannitol, 200 mM NaCl, 10 mM KCl, 1.2 mM $Mg(NO_3)_2$, and 20 mM Tris-HEPES, pH 7.6 using increasing concentrations of N-methylfurosemide.

Table 3.--Binding of N-methylfurosemide to rectal gland plasma membrane vesicles

Ion Dependence	Na		K		Cl	
	0 mM	200 mM	5 mM	50 mM	210 mM	610 mM
	49 ± 5	100	46 ± 2	100	100	47 ± 14
	n=4		n=4		n=3	
Competition	10^{-3}M Bumetanide		10^{-3}M Furosemide			
	44.5 ± 5.5		23.3 ± 10.0			
	n=3		n=3			

For experiments concerned with ion dependence, incubation media contained 0.8×10^{-6} M N-methylfurosemide, 100 mM mannitol, 20 mM Tris-HEPES, 1.2 mM $Mg(NO_3)_2$, 200 mM salt (Na Cl + KCl), pH 7.6 except in Cl experiments where 200 to 600 mM NaCl in the presence of 10 mM KCl was used. For experiments concerned with competition the incubation media contained 0.8×10^{-6} M N-methylfurosemide, 100 mM mannitol, 20 mM Tris-HEPES, 1.2 mM $Mg(NO_3)_2$, 200 mM NaCl, 10 mM KCl and 10^{-3} M bumetanide or furosemide, pH 7.6. Values are means ± standard error of the mean values derived from n experiments.

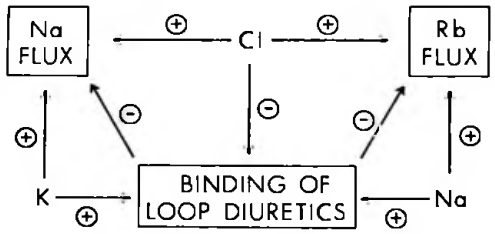

Figure 2.--Schematic representation of the interactions between the postulated Na, K, Cl-cotransport system and sodium, potassium (rubidium), chloride and loop diuretics.

obtained in these studies. Sodium flux can be inhibited by loop diuretics and is stimulated by chloride and rubidium. Rubidium flux is similarily inhibited by loop diuretics and stimulated by chloride and sodium. N-methylfurosemide binding is stimulated by sodium and potassium and inhibited by chloride. The latter results are similar to the report of Forbush and Palfrey (Biophys. J. 37:161/a, 1982). The experimental findings can be explained by assuming that

the plasma membranes contain a sodium, potassium, chloride cotransport system. The transport system has sodium, potassium and chloride binding sites, the latter being shared with the loop diuretics.

Thus the model for the rectal gland as proposed by (Silva and coworkers, Am. J. Physiol. 233:F298-306, 1977) can now be modified. Chloride secretion involves first accumulation of chloride inside the cell against its electrochemical potential via a sodium, potassium, chloride cotransport system located in the basal lateral membrane. Sodium extrusion from the cell via Na-K-ATPase results in a favorable gradient for sodium which provides the predominant driving force for the sodium, potassium, chloride cotransport system. Potassium recycling would occur via passive efflux through barium sensitive potassium channels (Silva et al. Bull. MDIBL 21:12-13, 1982) located in the basal lateral membrane. Chloride movement into the lumen would then occur via a sodium independent mechanism which may or may not be linked to potassium.

THE RENAL AND BRANCHIAL HANDLING OF CO_2/HCO_3^- IN THE MARINE ELASMOBRANCH

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CO_2 and HCO_3^- physiology in marine fish differs from that in terrestrial vertebrates. (W.W. Smith, J. Cell. Comp. Physiol. 14:95, 1939) and (Hodler et al (Am. J. Physiol. 183:155, 1955) showed that urine of the marine elasmobranch and teleost has no measurable HCO_3^- and a fixed pH of 5.7-5.8. These species lack renal carbonic anhydrase. Furthermore attempts to alter urinary pH by bicarbonate loading and/or carbonic anhydrase inhibition (Hodler et al., 1955: Boylan et al., Bull MDIBL 13:17, 1973; Murdaugh and Robin, in Sharks, Skates and Rays, 249, 1967 and Swenson et al., this bulletin) or nonbicarbonate base loading (Cohen, J. Cell. Comp. Physiol. 53: 205, 1959 and Swenson et al., this bulletin) have been notably unsuccessful. These fish avidly reabsorb HCO_3^- and maintain an acid urine in order to prevent precipitation of Mg^{++} and Ca^{++} salts (Smith, 1939). The lack of any significant renal acid base regulation in these species thus directed attention to the gills. Hodler et al., 1955 showed that HCO_3^- excretion occurs across the gill and this was confirmed by Murdaugh and Robin (vide supra) in more definitive experiments. This complex organ serves at least four crucial functions: acid-base regulation, nitrogenous waste excretion, NaCl homeostasis and gas exchange. We wished to explore in more detail elements of both the renal and branchial handling of HCO_3^- and CO_2 in the dogfish Squalus acanthias.

METHODS--Male sharks weighing 2 kg were caught by net in Frenchman Bay, Maine and kept in live cars until used. During the experiment they were placed in small boxes with free-flowing seawater (T=15-16°). The urinary papilla and dorsal artery were cannulated with PE-90 polyethylene tubing and the fish restrained in a normal orientation by the use of two wide soft encircling sponge rings that fit snugly in the box and around the fish. Blood pressure was monitored continuously and arterial blood samples drawn aneorobically for measurement of pO_2, pH, pCO_2, inulin and carbonic anhydrase inhibitor concentrations. Only those fish whose blood pressure remained stable (> 30 mm Hg) and arterial pO_2 > 90 mm Hg were used. Urine was collected for measurement of flow rate, pH, inulin, total CO_2 and titratable acid. pO_2 and pH were measured on a standard blood gas analyzer. Plasma and urine total CO_2 were measured manometrically and pCO_2 was calculated from the Henderson-Hassalbalch equation using a pk of 6.1 (Maren, Bull. MDIBL, 11:63, 1971) and a factor of 0.045, for the CO_2/HCO_3^- system. Urine titratable acid was measured by titration of 1 ml of urine to pH 7.8 with 0.1 N NaOH. GFR was measured by use of 2 μci of 14-C-inulin injected at the beginning of the experiment. The experimental design included a two hour period of baseline measurements after the inulin was given, followed by a two hour infusion of 30 m mole of $NaHCO_3$ (15 ml/hr/kg of 1M $NaHCO_3$) and a post infusion period lasting 4 hours. In certain experiments, at the conclusion of the bicarbonate infusion either benzolamide (1 mg/kg) or methazolamide (30 mg/kg) was given.