cell membrane because of a steep electrochemical gradient opposing it, but must enter the lumen through the paracellular space. The present experiments were designed to test the accuracy of the last statement. 2.4.6-triaminopyrimidine (TAP) was used as a probe of the paracellular pathway (Moreno, Nature. 251:150-151, 1974) in isolated perfused rectal glands.

Rectal glands were perfused as previously described (Silva et al. Am. J. Physiol. 233:F298-F306, 1977) with shark Ringers containing glucose 5 mM, 2.5×10^{-4} M theophylline and 5×10^{-5} M dibutyryl cyclic AMP at a pH of 6.7. This pH was chosen because TAP inhibits sodium flux via the paracellular pathway only in its protonated form and its pK is 6.72. At a pH of 6.7, 50% of the TAP in solution is protonated. The concentrations of TAP given in this report are those of the protonated form. At a pH of 6.7, the secretion of chloride by the stimulated isolated perfused rectal gland is 50 to 60% that during perfusion at a pH of 7.6, the normal pH of shark blood. Glandular secretion of chloride rapidly decreases as pH is lowered, until at a pH of 6.0, where 85% of any TAP present would be in the protonated form, it is less than 5% of normal.

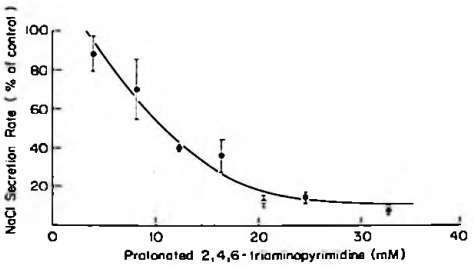

Figure 1.--Effect of 2.4.6-triaminopyrimidine on sodium secretion by the perfused rectal gland. The percent inhibition of sodium chloride secretion is plotted against the concentration of protonated TAP. Half maximal inhibition is found at about 11 mM. Inhibition is maximal at concentrations greater than 20 mM.

Figure 1 shows the effect of increasing concentrations of TAP on sodium chloride secretion by the stimulated shark rectal gland. The results are expressed as a percent of the rate of secretion in the absence of TAP. The half maximum inhibition was found at a TAP concentration of 12 mM. The effect was partially reversible. In glands perfused at concentrations of TAP of less than 16 mM it was always reversible. At 32 mM it was reversible only in 3 out of 9 glands.

The effect of TAP on rectal gland secretion is consistent with the view that sodium enters the lumen via the paracellular pathway. The inhibitory effect of TAP on rectal gland secretion was seen at concentrations 2 to 3 times higher than those at which it caused inhibition in the gallbladder or frogskin due perhaps to the fact that the sodium concentration in the perfusate of the rectal gland was two and a half times greater than that used in those experiments.

STOICHIOMETRY OF SODIUM CHLORIDE TRANSPORT BY THE SHARK RECTAL GLAND

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Previous work from our laboratory has shown that the ratio between chloride secreted and oxygen consumed by the rectal gland is approximately 25 to 30:1, greatly in excess of the 18:1 that can be calculated if 3 moles of sodium are transported per mole of ATP hydrolyzed by Na-K-ATPase in the basolateral membrane of the cells and the P:O ratio of the rectal gland mitochondria is 3:1 (Silva et al., J. Membrane Biol. 1980; 53:215-221). This has suggested, either that more than one chloride accompanies each sodium into the cell or that there is additional energy available from anaerobic metabolism. The latter explanation seems unlikely because lactic acid production by the gland is minimal.

To determine the stoichiometry of chloride and sodium in the process of stimulated secretion of chloride by the rectal gland we have studied the effect of progressive changes in the concentrations of chloride and of sodium in the medium used to perfuse isolated rectal glands.

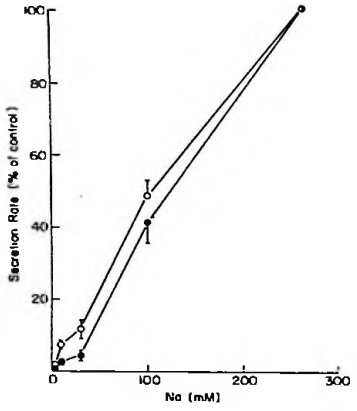

Figure 1.--Relation between the rate of secretion of sodium (●) and chloride (○) against sodium concentration in the perfusate, in six rectal glands. Sodium was replaced isotonically by N-methyl-D-glucamine. The rate of secretion is expressed as the percent of that secreted at a normal sodium concentration (280 mM).

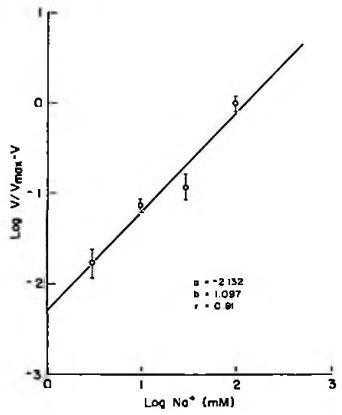

Figure 2.--Hill plot of the relation between the rate of chloride secretion versus the sodium concentration shown in Figure 1. The Hill coefficient is 1.097 not significantly different from one indicating that there is a single site for the interaction of sodium with the chloride transport system.

Rectal glands were perfused as previously described (Silva et al., Am. J. Physiol., 1977; 233:F298-F306). For isotonic substitution of sodium, N-methyl-D-glucamine was used and for chloride, either gluconate or nitrate was substituted. Each experimental protocol was started and ended with three or more consecutive ten minute periods of perfusion with a medium of normal sodium and chloride composition. Successive changes, increasing or decreasing the concentration of sodium or chloride in the perfusate, were made after three or more ten minute collection periods at each concentration. All the perfusion media contained in addition theophylline 2.5×10^{-4}M and dibutyryl cyclic AMP 5×10^{-5}M.

Sodium substitution studies

Figure 1 shows the relation between the rate of chloride secretion as a percent of that secreted at a normal sodium concentration (280 mM) in the perfusate, plotted against the sodium concentration in the perfusate, in six rectal glands. The concentrations of sodium in the perfusate were 3, 10, 30, 100 and 280 mM. The secretion of sodium and chloride follow essentially identical curves and no saturation is observed within the range of sodium concentration studied. A Hill plot of chloride secretion versus the sodium concentration (Figure 2) discloses a straight line with a slope of 1.097, not significantly different from 1, $p < 0.001$, indicating that there is only one site for the interaction of sodium with the chloride transport system.

Chloride substitution studies

Gluconate--A different pattern was seen when gluconate was substituted for chloride (Figure 3). The concentrations of chloride used were 3, 5, 7.5, 10, 20, 30, 60, 100 and 280 mM. The curves are sigmoidal showing saturation of the transport system at concentrations of chloride greater than 100 mM. A lineweaver-Burke plot of the chloride secretion rate versus the chloride concentration is curvilinear with an upwards concavity, indicating positive cooperativity (Figure 4). This suggests that when chloride interacts with the chloride transport system it

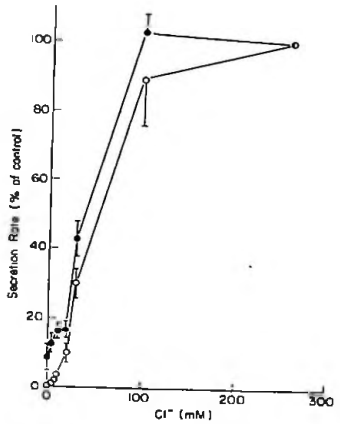

Figure 3.--Relation between the rate of secretion of sodium and chloride against the chloride concentration in the perfusate in twelve rectal glands. Symbols as in Figure 1. Chloride was isotonically replaced by gluconate. The rate of secretion is expressed as the percent of that secreted at normal chloride concentration (280 mM).

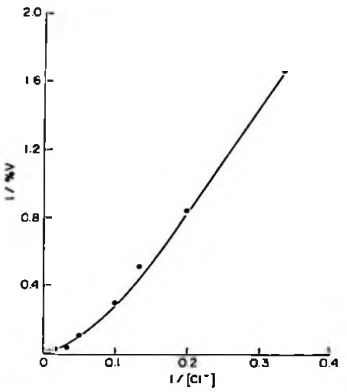

Figure 4.--Lineweaver-Burke plot of the data shown in Figure 3. The relation between chloride secretion and chloride concentration is curvilinear with an upwards concavity indicating positive cooperativity.

does so by way of separate sites with different affinities. A Hill plot of the rate of chloride secretion versus the concentration of chloride is shown in Figure 5. The slope of the line is 1.87, $p < 0.001$, indicating that there are at least two sites for the interaction of chloride with the chloride transport system.

Nitrate--The effect of substituting nitrate for chloride on chloride and sodium excretion is shown in Figure 6.

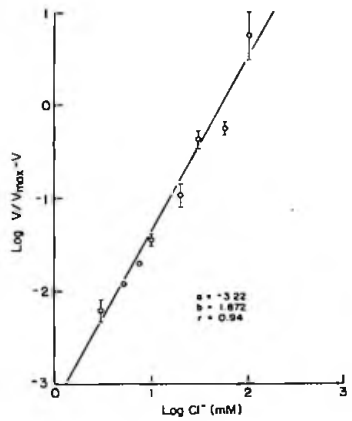

Figure 5.--Hill plot of the relation between the rate of chloride secretion and the chloride concentration shown in Figure 3. The Hill coefficient is 1.87 indicating that chloride interacts with at least two sites in the chloride transport system.

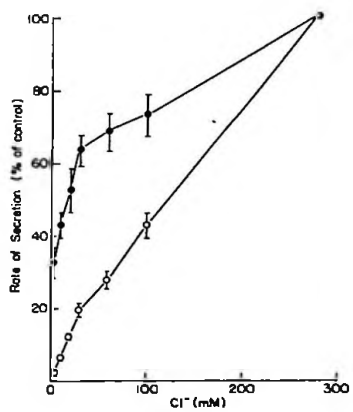

Figure 6.--Relation between the rate of secretion of sodium and chloride against the chloride concentration in the perfusate in six rectal glands. Chloride was replaced isotonically by nitrate. Symbols as in Figure 1. The rate of sodium secretion exceeds that of chloride indicating that there is transport of nitrate. At similar chloride concentration the rate of sodium and chloride secretion is lower than that seen with gluconate, chloride lower than sodium, indicating that nitrate is also inhibiting transport.

The pattern of chloride secretion is quite different from that of sodium. The rate of chloride secretion represents a variable fraction of the secretion of sodium, that changes with the concentration of chloride in the perfusate, indicating that a variable proportion of nitrate is transported by the rectal gland together with sodium. A Hill plot of

the chloride secretion rate versus chloride concentration is depicted in Figure 7. The slope of this relation is 1.0, $p < 0.001$, indicating that in the presence of nitrate only one chloride interacts with the transport system. Consistent with this inference, a Lineweaver-Burke plot of the rate of chloride secretion versus the concentration of chloride in the perfusate is linear (Figure 8). These findings suggest also that nitrate can replace chloride at one of its sites of interaction with the contransport carrier.

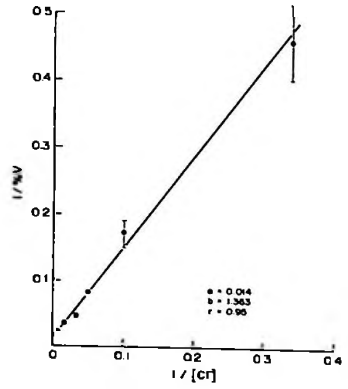

Figure 7.--Hill plot of the relation between chloride secretion and chloride concentration shown in Figure 6. The Hill coefficient is 1.0 indicating that in the presence of nitrate chloride interacts with only one site in the chloride transport sytem.

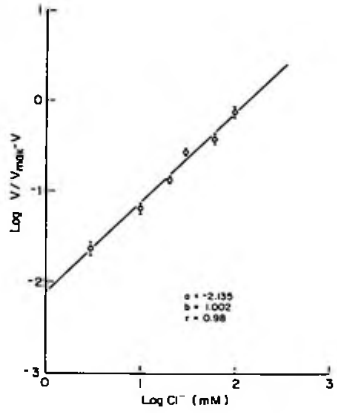

Figure 8.--Lineweaver-Burke plot of the chloride secretion data shown in Figure 6. The relation is linear. A Km of 100 μM for chloride can be calculated suggesting that nitrate is interacting with the high affinity site.

These findings provide strong support for the simultaneous interaction of $1Na^+$ and $2Cl^-$ with the rate-limiting co-transport carrier in stimulated secretion by the rectal gland. A similar mechanism, presumably involving $1\ Na^+$, $1K^+$ and $2Cl^-$, probably is present in other cells, including flounder intestinal mucosa, Erlich ascites tumor, avian erythrocytes, and the mammalian thick ascending limb. Unexpectedly, nitrate, but not gluconate, appears to have a limited ability to substitute for chloride on one of its carrier sites, and this may prove to be the case in other chloride transporting systems as well.

ANTIPYRYLAZO III MEASUREMENTS OF EXTRACELLULAR Ca^{2+} DEPLETION IN VOLTAGE CLAMPED FROG VENTRICULAR MUSCLE

Lars Cleemann, JoAnna S. Choo and Martin Morad, University of Pennsylvania, Department of Physiology, Philadelphia, Pa.

Ca^{2+} for activation of tension in cardiac muscle cells is thought to depend in part on transport of Ca^{2+} across the membrane and in part on the release of Ca^{2+} from intracellular pools. To assess the role of extracellular Ca^{2+} in immediate activation of tension we decided to investigate whether Ca^{2+} enters the cell in sufficient quantity to allow measurement of its depletion from the extracellular space. For this purpose we have developed an optical technique which utilizes the color change produced by antipyrylazo III, an impermenant Ca-sensitive dye. Preliminary results show that a Ca depletion signal can be measured after careful elimination of contraction artifacts. This signal has a wavelength dependency predicted by spectrophotometric measurements of free dye in Ca^{2+}-containing solutions. The Ca^{2+}-depeltion signal appears to include a component which might reflect the slow inward current. It was concluded that the development of the optical technique to measure Ca^{2+} movement across the membrane provides data on the kinetics of Ca^{2+} movement across the membrane not easily obtained with much slower Ca^{2+}-sensitive electrodes.