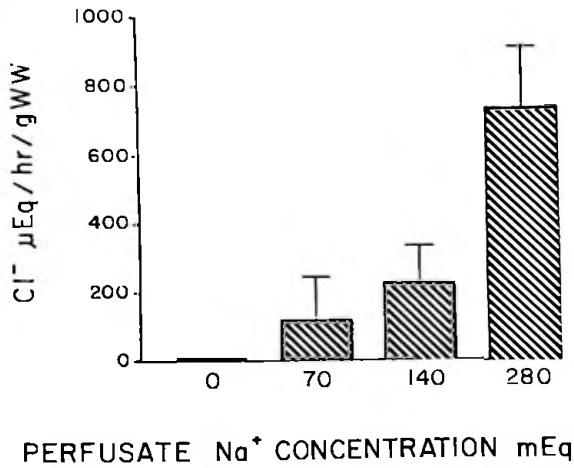

Figure 2. The effect of increasing progressively the concentration of sodium in the perfusate from 0 to 70, 140, 280 mM on chloride secretion rate in five rectal glands in vitro. There is a linear relation between the rate of chloride secretion and the concentration of sodium in the perfusate.

The possible effect of nonspecific cation substitution was then tested by substituting Tris for sodium. When Tris replaced sodium in the medium perfusing the stimulated rectal gland, chloride secretion decreased from 1667.5 ± 276.5 to 133.7 ± 35.6 µEq/hr/g wet weight. Restoring sodium concentration to 280 mM increased chloride secretion to 1403.9 ± 123.2 µEq/hr/g wet weight just as in the experiments with choline chloride.

The sodium-dependent chloride secretion exhibited by the stimulated rectal gland is reminiscent of the sodium-dependent electrolyte transport reported for mammalian intestine, pancreas, salivary gland, cornea, gastric mucosa, and frog skin. Similarly, other transport systems responsible for the reabsorption of glucose and amino acids are known to require sodium. The sodium-dependent chloride secretion observed in the rectal gland is one of the features that characterize a general hypothesis for chloride transport that has been previously proposed. The movement of chloride into the cell against an electrical gradient is coupled to the passive toward movement of sodium down its electrochemical gradient. Sodium and chloride move across the basolateral cell membrane via coupled facilitated diffusion. Maintenance of the gradient facilitating the movement of sodium into the cell is due to the continued activity of Na-K-ATPase located in the basolateral membrane of the cell, hence the inhibition of chloride secretion by ouabain. Movement of chloride across the luminal membrane of the cell is believed to be passive along an electrical gradient.

INTRACELLULAR CYCLIC AMP LEVELS AND ACTIVE CHLORIDE TRANSPORT IN THE RECTAL GLAND OF *Squalus acanthias*: THE EFFECT OF VASOACTIVE INTESTINAL PEPTIDE

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The rectal gland of *Squalus acanthias* is a compound tubular gland which actively transports chloride. Previous work from our laboratory utilizing an in vitro perfusion technique has established

that chloride transport is stimulated by theophylline and dibutyryl cyclic AMP. The results of these experiments were consistent with the view that active transport was mediated by changes in the intracellular concentration of cyclic AMP, although more direct evidence was lacking. The present studies were conducted to test this hypothesis and to investigate the factors which may regulate cyclic AMP metabolism in the rectal gland epithelial cell.

Spiny dogfish of either sex weighing 2 to 5 kg, were captured and maintained in the usual manner. Rectal glands were removed and placed in well oxygenated dogfish Ringer at room temperature (20 ± 5°C). The gland was serially sectioned along a coronal plane into 5 mm segments. Slices 0.5 mm in thickness were then prepared with the use of a Stadie-Riggs microtome (usually 15-25 slices per gland). Slices were then transferred to either 25 ml Erhlenmeyer flasks or 5 ml polypropylene vials which contained 5 ml or 1.5 ml of well oxygenated dogfish Ringer respectively. The concentration of all additions are given in the legend or text. Slices were then incubated at room temperature for the times indicated. After incubation the slice was rapidly removed and plunged into liquid nitrogen (-180°C) within 5 sec of termination of the reaction. The tissue was powdered in a porcelain mortar and transferred into 1 ml of iced 10% trichloroacetic acid (TCA) containing tracer quantities of ^{3}H-cyclic AMP (10^{5} CPM). Cyclic AMP was extracted by thorough vortexing and a freeze-thaw step. The extract was centrifuged at 5000 rpm for 10 min at 4°C and the supernate aspirated. The precipitate was extracted again with a second ml of 10% TCA in a similar manner and the resultant supernate was pooled with the initial supernate. The cell pellet was digested in 0.5 ml of 1 N NaOH and the concentration of protein determined. The pooled supernates were acidified with 0.1 ml of 1 N HCl and purified by column chromatography with analytical grade cation exchange resin in the hydrogen ion form. Column eluates (6 ml) were concentrated by flash evaporation at 70°C under vacuum and resuspended in 0.2 ml of 50 mM sodium acetate buffer pH 4.0. Samples were then assayed by protein kinase binding assay. All samples were analyzed in duplicate usually performed at two or more dilutions and the values expressed as the mean ± SE. All cyclic AMP values were corrected for recovery of the added ^{3}H-cyclic AMP and recoveries varied from 60-90%.

Theophylline is a potent stimulus of chloride ion transport in the rectal gland. Figure 1 illustrates that theophylline also increases intracellular concentrations of cyclic AMP in a similar dose-dependent fashion. The increment in cyclic AMP concentration was measured at 5 min after incubation with the drug. This effect however, occurs even more promptly and may be seen as early as 30 sec after the addition of the theophylline (5 mM) as depicted in Figure 2. The rise in the intracellular concentration of the nucleotide is not sustained and tissue levels fell to baseline values within 15 min, despite the continued presence of this cyclic nucleotide phosphodiesterase inhibitor in the media.

In order to further assess the relationship between the theophylline induced cyclic AMP response and stimulation of active chloride transport, experiments were performed in media in which sodium was replaced in equimolar amounts by tris(hydroxymethyl)amino-methane (Tris) chloride. Previous experiments by our laboratory (reported in this Bulletin) have demonstrated that sodium transport is tightly coupled to active chloride movement. Table 1 illustrates the relationship between the rate of active chloride transport and the rate of cyclic AMP accumulation as extracellular sodium concentration is varied. The data presented for chloride transport are derived from in vitro perfusion studies. The rate of chloride transport and intracellular cyclic AMP accumulation varied directly with extracellular sodium concentration. This suggests that the

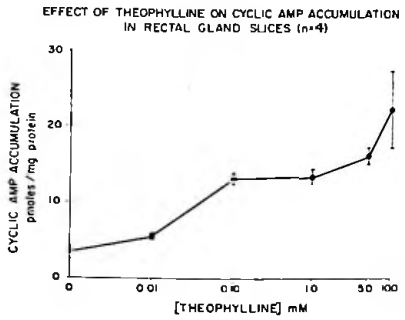

Figure 1. Effect of theophylline on cyclic AMP accumulation in rectal gland slices (n=4)

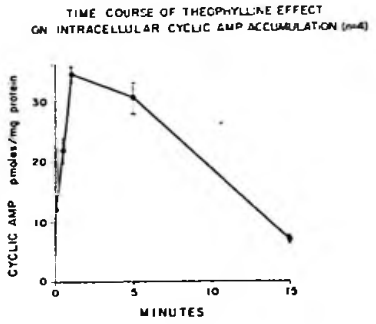

Figure 2. Time course of theophylline effect on intracellular cyclic AMP accumulation (n=4)

TABLE 1

Effect of Extracellular Sodium Concentration on Rate of Active Chloride Transport and Intracellular Cyclic AMP Accumulation

Na^+ (mM)	Cl^- Transport µEq/hr/gWW	Cyclic AMP pmoles/mg protein
0	0	8.43
70	122.0	10.25
140	220.8	13.62
280	735.2	36.31

Theophylline (1 mM) was present in all experiments. Data for chloride transport (n=5) and cyclic AMP (n=2) are mean values.

intracellular level of cyclic AMP may have an important regulatory function on chloride transport rate. Further evidence in support of this important relationship is the temporal nature of the theophylline induced increase in active chloride transport and cyclic AMP accumulation. Both intracellular cyclic AMP and chloride transport rate rise in parallel fashion and peak between 5 and 10 minutes. The fall in cyclic AMP is then followed by a subsequent decline in active chloride transport rate.

The observation that intracellular cyclic AMP is an important factor in regulating active chloride ion transport rate has stimulated an intense search for the humoral or neurohumoral factor(s) for which the cyclic nucleotide was the second messenger. No effect was seen with vasopressin and several oxytocin derivatives (10^{-6} M) normally found in the posterior lobe of the pituitary of *Squalus acanthias*, norepinephrine (6×10^{-6} M), epinephrine (6×10^{-6} M), epinephrine (5×10^{-5} M), serotonin (10^{-4} M), substance P (2 ng/ml) and calcitonin (100 µg/ml). Since the rectal gland is an appendage of the intestine it seemed possible that hormones which stimulated intestinal secretion might have an effect on this organ. Accordingly glucagon (1 ng/ml),

secretin (75 U/ml) and vasoactive intestinal peptide (1.6×10^{-6} M) were investigated by the in vitro isolated organ perfusion technique. Neither secretin nor glucagon had any effect over the concentration range studied. Vasoactive intestinal peptide (VIP) had a marked stimulatory effect on active chloride transport producing a greater than 500% increase in transport compared to control perfusions (230 ± 51.3 to 1222 ± 304 μEq/hr/gWW, $p < 0.01$, n=6).

Additional studies were carried out to determine whether this concentration of VIP produced a concomitant increase in intracellular cyclic AMP concentration. VIP produced a doubling of intracellular cyclic AMP compared to controls (15.4 to 29.8 pmoles cAMP/mg protein) and was increased further by theophylline (1 mM) (27.7 to 94.8 pmoles cAMP/mg protein). Thus this hormone increases active chloride transport and intracellular cyclic AMP accumulation and is synergistic with theophylline. Additional experiments were carried out with the intact fish to establish the presence of the hormone and define the factors which regulate its release. These results are not available at the present time.

In summary, active chloride transport in the rectal gland of the spiny dogfish is tightly coupled to the intracellular level of cyclic AMP. Cyclic AMP concentration is regulated in part by the activity of the degradative enzyme, cyclic nucleotide phosphodiesterase which is inhibited by theophylline. In addition there appears to be a feedback mechanism which either alters the activity of the diesterase or inhibits adenylate cyclase activity resulting in a fall of the intracellular level of the nucleotide. Furthermore our studies indicate that extracellular sodium concentration modifies the cyclic AMP response to theophylline. Our studies with VIP demonstrate that this hormone activates the gland in vitro resulting in an increase in cyclic AMP production and active chloride transport. Further studies are required to establish whether this hormone plays an important regulatory role in the intact fish.

AN INTRODUCTION TO COMPARATIVE STUDIES OF THE RATE OF THE BOHR EFFECT

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The Bohr effect describes the protonation of HbO_2, which elicits a conformational change in the protein, leading to release of O_2. It yields a "shift to the right" in the oxygen dissociation curve, meaning that pO_2 increases with acidosis, at any level of oxygen saturation. The rate of the reaction $H^+ + HbO_2 \rightarrow HHb + O_2$ is extremely rapid, half-time being of the order of 10 milliseconds. The rate of the Bohr shift is thus determined by the rate of formation or appearance of H^+ within the red cell.

Our interest in this subject was effected by the finding of R. E. Forster and J. B. Steen (J. Physiol., 196:541, 1968) that carbonic anhydrase inhibition reduced the rate of the CO_2-mediated Bohr shift in human red cells by about thirty-fold. This corresponds well to a model in which the protonation of hemoglobin is linked to the hydration of CO_2 in tissue capillaries. Thus the formation of the two chief proteins (hemoglobin and carbonic anhydrase) in red cells is linked.

We have begun a study of the Bohr shift, with the following questions in mind: (1) What are the quantitative relations between inhibition of carbonic anhydrase and rate of the Bohr shift? (2) Are there physiological and clinical sequelae to marked reduction in rate of the Bohr shift? (3) Does the magnitude of the Bohr shift in vertebrate and invertebrate species have any relation to carbonic anhydrase activity of the blood?