chloride as well as their independent exit (alternative (i) above), then the addition of furosemide to block this entry should lead to a fall in tissue sodium and chloride levels. From the data presented in Figure 2, it is clear that no such decline in tissue sodium and chloride levels occurs and that in fact the addition of 2.5×10^{-4} M furosemide produces no significant change in tissue ions in either control or cAMP-treated tissue.

It is concluded that the results of these preliminary experiments indicate that a major action of cAMP in the rectal gland is the stimulation of sodium entry into the cells and furthermore that this stimulated sodium uptake is largely independent of any mechanism of chloride uptake. We suggest therefore that, contrary to current models, any furosemide-inhibited coupled sodium-chloride co-transport system can be of only minor significance in the overall mechanism of cAMP-stimulated secretion in the dogfish rectal gland.

This work was supported by grants from the U.K. Science Research Council (GR/A29494) and the Marshall and Orr Bequest of The Royal Society, London.

OUABAIN-BINDING IN THE RECTAL GLAND OF *Squalus* - THE EFFECTS OF CYCLIC AMP, SODIUM AND FUROSEMIDE

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In slices of the rectal gland of the European dogfish, *Scyliorhinus canicula*, the presence of dibutyryl cyclic AMP (cAMP) and theophylline in the incubation saline produces a marked increase in ouabain binding (Shuttleworth and Thompson, J. exp. Zool. 206:297-302, 1978). However, attempts using both perfused preparations (Silva *et al.*, Bull. MDIBL 18:16-19, 1978) and tissue slices (Epstein, personal communication) have failed to demonstrate a similar increase in glands from *Squalus acanthias*. This apparent discrepancy was investigated with a view to determining whether it represented a species difference or was the result of a difference in the techniques employed.

Rectal gland tissue was taken, sliced and incubated in a manner essentially similar to that described previously (J. exp. Zool. 206:297-302, 1978). The chief differences were that the incubating temperature was 15°C and that the total concentration of ouabain in the incubation medium was 2.25×10^{-6} M containing ^{3}H ouabain at 0.25 µCi ml^{-1}. Tissue samples were removed from the incubation medium, given three five-minute washes in ice-cold ouabain-free saline and blotted dry. Following over-night drying at 95°C, the samples were weighed, solubilized (Soluene, Packard) and the degree of ouabain binding determined by liquid scintillation spectrometry. All results were expressed as pmol ouabain bound per mg dry weight (± S.E.).

Preliminary experiments were run to determine the time required for complete labeling of available sites in the tissue. No significant increase in binding occurred beyond 3 to 4 hours, so an incubation time of 4 hours was used in all subsequent experiments. The results are presented in Table 1.

The degree of ouabain binding in tissue from *Squalus* incubated in normal dogfish Ringer was very similar to that obtained previously for *Scyliorhinus* rectal gland (18.3 ± 1.3 pmol mg dry^{-1}). Incubation in 0.05 mmole l^{-1} cAMP and 0.25 mmol l^{-1} theophylline produced an increase of approximately 19% in the degree of ouabain binding which was statistically significant ($t = 2.574$, $P < 0.05$). This increase is clearly very much less than the 87% increase found in *Scyliorhinus* but indicates that the difference between the two species in this respect is quantitative rather than qualitative.

An intriguing aspect of these experiments is the long time periods required for complete ouabain binding (2 hours in *Scyliorhinus*, 4 hours in *Squalus*) compared to the very rapid effects of ouabain on

secretion (Silva *et al.*, Am. J. Physiol. 233:298-306, 1977) and oxygen consumption (Shuttleworth and Thompson, J. comp. Physiol.: in press). A ouabain concentration of 2.25×10^{-6} M is sufficient to virtually completely inhibit the Na^+-K^+-ATPase from *Squalus* rectal glands (Bonting, Comp. Biochem. Physiol. 17:953-966, 1966), so an obvious effect of the incubation in these experiments would be the inhibition of the sodium pump and the subsequent progressive loading of the intracellular environment with sodium. To determine whether sodium loading of the intracellular environment was in any way responsible for the development of the final levels of ouabain binding observed, the experiment was repeated under conditions of minimal sodium loading. To achieve this, sodium chloride in the Ringer was replaced by choline chloride to give an external sodium concentration of 2.5 mmol l^{-1}. Assuming an intracellular potential of between -60 mV and -80 mV, an external sodium concentration of 2.5 mmol l^{-1} would be in equilibrium with internal concentrations of 28-73 mmol l^{-1}, and in the absence of precise measurements enabling exact electrochemical gradients to be determined, it was considered that, under such conditions, net sodium entry following inhibition of the sodium pump would be minimal. It can be seen from Table I that ouabain binding in this situation is reduced to only 27% of that measured in normal Ringer, and it is concluded that net sodium entry is in some way modifying the level of ouabain

TABLE I

Ouabain Binding in Rectal Gland Tissue Slices

Incubation Medium	Ouabain Binding pmol mg dry wt^{-1}
A. Normal Ringer	21.3 ± 1.3 (6)
B. Normal Ringer + cAMP/theophylline	25.4 ± 2.2 (6)
C. Low Na^+ Ringer	5.8 ± 0.4 (5)
D. Normal Ringer + furosemide	16.3 ± 1.4 (5)

All values expressed as mean ± S.E. (N). Medium B contained 0.05 mmol l^{-1} cAMP and 0.25 mmol l^{-1} theophylline. In medium C, NaCl was replaced with choline chloride giving a final Na^+ concentration of 2.5 mmol l^{-1}. Medium D contained 5×10^{-4} M furosemide.

binding in this tissue. One explanation of this is that the cell membranes, both in control and cAMP-stimulated tissues, contain what may be called "latent sites" for the binding of ouabain and that these sites depend on net sodium entry into the cells for their manifestation or activation. Such a hypothesis would also explain the fact that cAMP produces a relatively small and progressive increase in ouabain binding but a very large and very rapid increase in ouabain-sensitive oxygen consumption (Shuttleworth and Thompson, J. comp. Physiol.: in press) and secretion rate (Stoff *et al.*, J. exp. Zool 199:443-448, 1977). Thus, it has been shown that an important effect of cAMP in the rectal gland is to greatly increase sodium entry into the cells (Shuttleworth and Thompson, this bulletin). It is envisaged that this would lead to a temporary loading of the intracellular environment with sodium which would, according to our model, activate the "latent sites" in the membrane producing a rapid increase in pump activity. Subsequent to this, cAMP would have its additional, long-term effect of an increase in the number of sites *per se* and eventually an equilibrium would be attained with the increased rate of sodium entry being balanced by the increased overall activity of the pump. Further investigations of this "latent site hypothesis" are clearly warranted.

In the previous report, we have produced evidence that most, if not all, of the cAMP-stimulated entry of sodium into the cells of the rectal gland is independent of any simultaneous chloride uptake and is not inhibited by 5×10^{-4} M furosemide. It has been shown above that the level of ouabain binding in the rectal gland is highly dependent on the simultaneous net entry of sodium following inhibition of the sodium pump. It was therefore thought to be of interest to test for any furosemide-sensitivity in the sodium entry process by investigating the effect of furosemide on ouabain binding. As can be seen from Table I, 5×10^{-4} M furosemide produced only a 23% reduction in ouabain binding compared to the 77% reduction in conditions of minimal sodium loading. This further emphasizes the conclusion reached in the previous report, that any furosemide-sensitive mechanism is only of minor significance in the overall sodium entry process in the dogfish rectal gland.

The financial assistance of the U.K. Science Research Council (GR/A29494) and the Marshall and Orr Bequest of The Royal Society, London, is gratefully acknowledged.

THE CLEARANCE OF METHAZOLAMIDE BY THE RECTAL GLAND, IN RELATION TO THE FAILURE OF CARBONIC ANHYDRASE INHIBITION TO ALTER SECRETION

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Although the rectal gland has the highest concentration of carbonic anhydrase of any tissue in *Squalus acanthias* (Maren, Physiol. Rev. 47:595, 1967) the function of the enzyme in this tissue has not been discovered. Among secretory organs containing carbonic anhydrase, the situation is unique, in that the sulfonamide inhibitors of the enzyme appear to have no effect upon fluid or NaCl output by rectal gland *in vivo* or *in vitro* (Rawls, Bull. MDIBL 4:58, 1962; Siegel *et al.*, Comp. Biochem. Physiol. 51A:1593, 1975, Silva *et al.*, Amer. J. Physiol. 233:T298, 1977).

We wished to find how methazolamide, a potent inhibitor with good access to mammalian tissue (Maren, *vida supra*) was handled by the rectal gland. The K_I of this drug for the rectal gland carbonic anhydrase is 2×10^{-7} M, very similar to what we found for dogfish red cells (Maren and Friedland, Bull. MDIBL 17:35, 1977). The isolated gland was perfused through its artery at 2 - 5 ml/min with dogfish Ringers solution containing 2×10^{-3} M methazolamide. The gland was stimulated by 0.25 mM theophylline and 0.05 mM dibutyrl cyclic AMP in the perfusion fluid in experiment of Table I and 0.25 mM dibutyrl cyclic AMP and 5 mM theophylline in experiment of Table II. The techniques have been described by Silva *et al.* (*vide supra*).

Tables I and II show the data. It is clear once again that methazolamide did not alter fluid production or chloride output by the gland, during periods in which there was a high concentration of the drug in the gland fluid. The clearance data, taken in the light of the study by Bradley and Georgopoulos (Bull. MDIBL 17:81, 1977) show a high degree of permeability for methazolamide across the rectal gland epithelium. The clearance of methazolamide is about 3 µl/min per g gland (normal gland weighs 3 g) which is 4 times greater than Bradley and Georgopoulos (*vide supra*) found for sucrose, and 10 times greater than their figures for inulin or ferrocyanide. If the drug in ductal fluid (59-270 µM) is in contact with tissue carbonic anhydrase, the concentration is approximately 300-1000 times the K_I. If we calculate on the basis of arterial perfusate or venous effluent (2 mM) the increment is about 10^4.

Further analysis of the experiment of Table II gives an estimate of methazolamide extraction by the gland. The total amount extracted from perfusate is the sum of drug (1) in the glandular fluid