K gluconate into vesicles pre-equilibrated for more than 12 hours in the same media. If Na entry occurred by a Cl-coupled process, then uptake should have been greater in KCl than in K gluconate. However, as shown in Figure 3b, Na uptake was the same in KCl and K gluconate. Therefore, the observed anion dependence cannot be explained by an obligatory NaCl symport. It is not clear why the previously observed (Eveloff et al., J. Comp. Phyiol. 135:175, 1980) furosemide-inhibitable Cl dependent Na influx was not observed in the present study. Differences in assay conditions may have been responsible; in the earlier study Na uptake was measured in the presence of 100 mM mannitol, 20 mM Tris-Hepes, 2 mM Ca gluconate at pH 8.2.

To determine if K is essential to NaCl symport (i.e., Na, K, Cl symport; see Musch et al., Bull. MDIBL, 1981), we compared Na uptake into TMACl (TMA is an impermeant cation) and KCl pre-equilibrated vesicles. If K facilitated NaCl transport a higher Na uptake would have been expected in Kcl. However, as shown in Figure 3b this is not the case; in contrast, there is higher Na uptake into TMACl vesicles, suggesting that K competes for either the H^+ gradient and/or the same transporter. The observation that Na uptake is higher in the presence of TMACl than TMA gluconate (Table 2) suggests that there is an anion-dependent, K-independent uptake but it remains to be established whether this is due to Cl/OH exchange or a Cl diffusion potential.

EFFECT OF STIMULATION OF THE RECTAL GLAND ON OUABAIN BINDING: EVIDENCE FOR A DIRECT ACTION OF CYCLIC AMP

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Chloride secretion by the rectal gland of the dogfish depends on the activity of Na-K-ATPase located in the basolateral membrane of the cells lining the tubules of the gland. When chloride secretion is stimulated by theophylline and cyclic AMP there is indirect evidence that Na-K-ATPase is simultaneously activated. First, ouabain inhibitable oxygen consumption increases six-fold; second, the intracellular concentration of sodium decreases while that of potassium rises. Both these findings are consistent with primary activation of Na-K-ATPase. However, the activity of Na-K-ATPase measured in homogenates in vitro was the same whether the glands were previously perfused with or without theophylline and cyclic AMP. Shuttleworth and Thompson (J. Exper. Zool., 206:297-302, 1978), reported that the binding of 2.2×10^{-6}M ouabain to the rectal gland of the dogfish Scyliorhinus canicola was increased after incubation with theophylline and cyclic AMP. Previous attempts in our laboratory to demonstrate increased binding of 5×10^{-6}M or 10^{-4}M ouabain to rectal gland slices of Squalus acanthias were unsuccessful (Bull. MDIBL 18:16-19, 1978; J. Cell Biol. 83:16-32, 1979). The present experiments were designed to investigate further the nature of the binding of ouabain to rectal gland cells.

All experiments were done using rectal glands from spiny dogfish, Squalus acanthias. The glands were removed via an abdominal incision and perfused by gravity with shark-Ringers for ten minutes to remove all red cells.

Coronal slices of rectal gland were prepared using a Stadie-Riggs microtome. The slices were kept in ice cold shark-Ringers until used, usually within fifteen minutes. The binding of ouabain to slices of rectal gland was measured in a solution containing 10^{-9}M carrier free ^{3}H-ouabain. A parallel incubation containing 10^{-3}M theophylline and 10^{-3}M dibutyrylcyclic AMP was run to determine the effect of stimulation of rectal gland secretion on ouabain binding. Additional incubations were done with varying concentrations of cold ouabain in order to determine the kinetics of ouabain binding. Nonspecific binding of ouabain to the slices was measured using 10^{-4}M cold ouabain. Extracellular space was labelled using ^{14}C-inulin, 0.1 microCi/ml. After varying times of incubation, the slices were removed from the incubation medium and transferred through

three successive washes in ouabain free solutions of the same composition as the incubation medium to remove all unbound ouabain. Adequacy of removal of the free ouabain was assessed by measuring the amount of ^{14}C-inulin remaining in the slices after the three washes. In all experiments ^{14}C counts returned to background. The slices were then lightly blotted on a piece of filter paper, weighed, and dissolved in NCS^{R}. The dissolved slices were counted for radioactivity in a scintillation counter using Spectrafluor^{R}. Specific binding to the slices was calculated from the radioactivity remaining in the tissue minus that remaining in the presence of 10^{-4}M ouabain, divided by the specific activity of the ouabain in the medium.

Cells were isolated from rectal glands using the technique previously described (MDIBL Bull. 20:38-39, 1980) with only minor modifications. The cells were kept on ice until used in a buffer of the following composition (in mM): Na 280; K 5; Cl 295; Mg 3; Ca 2.5; SO_4 0.5; phosphate 1; Urea 350; Hepes 40, pH 7.6. Binding of ouabain to isolated rectalgland cells was measured using a filtration technique. ^{14}C-Inulin, 0.1 microCi/ml, was used as a marker of extracellular space. Incubations were stopped by dilution of a measured amount of cells, still in the incubation medium, in ten times the volume of ouabain free incubation solution followed by rapid filtration through HA 0.45 micron Millipore filters. The filters were dissolved in 1 ml of ethylacetate and radioactivity counted in a scintillation counter using Hydrofluor^{R}. Non-specific binding of ^{3}H-ouabain to the cells was determined by displacement of the labelled ouabain with 10^{-4}M unlabelled ouabain. Non-specific binding to the filters was measured by filtering incubation solution containing labelled and unlabelled ouabain and labelled inulin but without cells. Specific binding to the cells was calculated from the total binding minus the non-specific binding after incubation with 10^{-4}M ouabain and minus the non-specific binding to the filter blanks.

Results are expressed as picomoles of ouabain bound per unit of wet weight in the case of the slices or milligram of protein in the case of the cells. Values are mean ± SEM.

Characteristics of ouabain binding

The time course of the binding of 10^{-9}M ^{3}H-ouabain to slices of rectal gland was quite slow requiring approximately five hours to reach a steady state (Figure 1). Binding of ^{3}H-ouabain was 50% greater in the

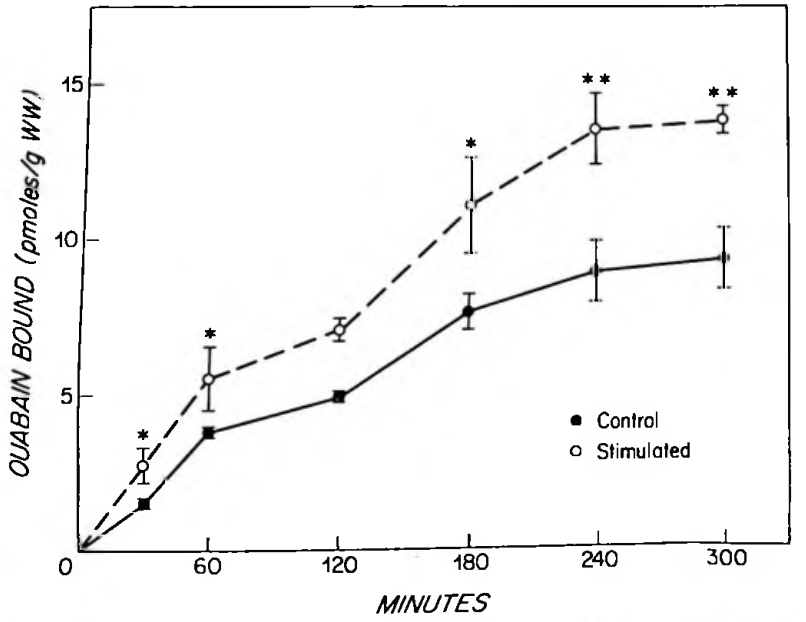

Figure 1.--Time course of ^{3}H-ouabain binding to slices of rectal gland. Each point represents duplicate determinations in four experiments. Steady state binding was reached only after a four hour incubation. Binding was 50% greater in the presence of theophylline 10^{-2}M and dibutyryl cyclic AMP 10^{-2}M. Symbols are mean ± SEM.

presence of theophylline and cyclic AMP than in the controls. The rate of labeling of the extracellular space with ^{14}C-inulin was measured in a separate set of slices to determine whether the slow binding of ouabain was due to poor permeation of the extracellular space by the ouabain. The number of ^{14}C-inulin counts reached a maximum between 30 and 60 min of incubation and remained stable thereafter indicating that there was rapid equilibration between the extracellular space of the slices and the incubation solution. The time course of binding was also measured in isolated rectal gland cells where the extracellular space is reduced to the basolateral membrane infoldings (Figure 2). These show virtually the same pattern as that seen with the slices.

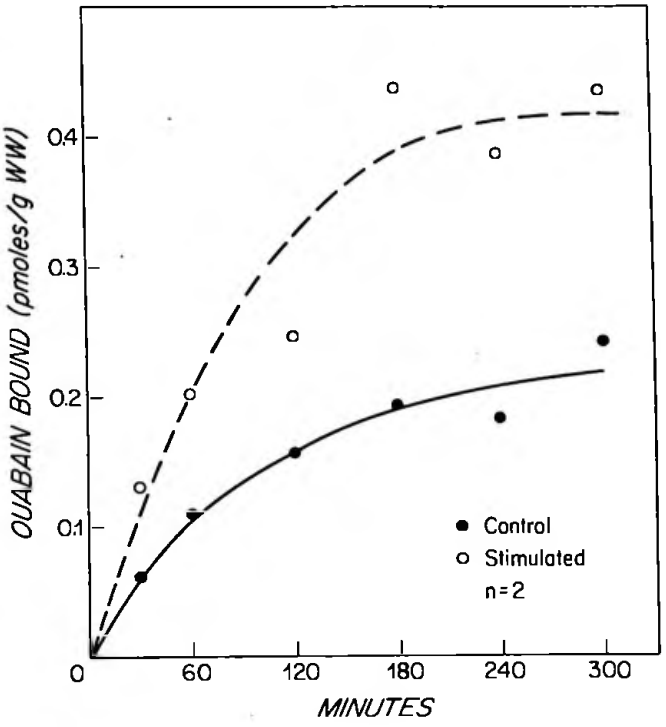

Figure 2.--Time course of ^{3}H-ouabain binding to isolated rectal gland cells. Each point represents single determinations in two experiments. The time course of binding was virtually the same as that with slices. Symbols are the average of two experiments.

The relation between ouabain bound to slices of rectal gland and ouabain concentration in the incubation solution is shown graphically in Figure 3. At concentrations of ouabain lower than 10^{-6}M, theophylline and cyclic AMP increase ouabain binding above that of controls. At concentrations of ouabain above 10^{-6}M the binding of ouabain is depressed by theophylline and cyclic AMP. At all concentrations of ouabain, except that at 10^{-6}M, the binding of ouabain in the presence of theophylline and cyclic AMP was significantly different from that of the controls. This is not apparent in Figure 3 because of the log/log nature of the plot. In isolated rectal gland cells the pattern of ouabain binding in relation to ouabain concentration was qualitatively the same. The decrease in the binding of ouabain seen at concentrations of ouabain greater than 10^{-6}M confirms previous observations in our laboratory (Bull. MDIBL 18:16-19, 1978).

Figure 4 shows a Lineweaver-Burk plot of the relation between ouabain bound and ouabain concentration. The relation between 1/ouabain bound vs 1/ouabain concentration is markedly curved particularly at the higher concentrations close to the origin of the curve which are not clearly seen at the scale shown here. A Lineweaver-Burk plot of the data obtained with isolated rectal gland cells was qualitatively similar.

A Scatchard plot of the data is shown in Figure 5. The relation between bound/free ouabain and bound ouabain is curvilinear both in the presence and absence of theophylline and cyclic AMP. A Scatchard plot of the binding of ouabain to isolated rectal gland cells was similar to the one for slices. Curvilinearity in both the

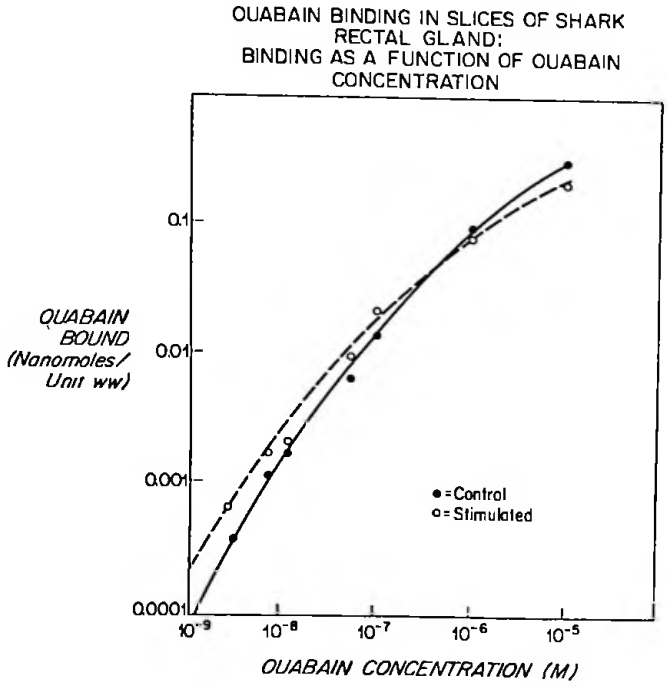

Figure 3.--Relation between ouabain concentration in the incubation solution and ouabain bound to slices of rectal gland. Each point represents duplicate determinations in four experiments. No measure of variance is shown because of the log/log nature of the graph. With the exception of the points at 10^{-6}M ouabain, stimulation with theophylline 10^{-2}M and dibutyryl cyclic AMP 10^{-2}M significantly changed ouabain binding to slices of rectal gland. Below 10^{-6}M it increased binding, above 10^{-6}M it reduced it.

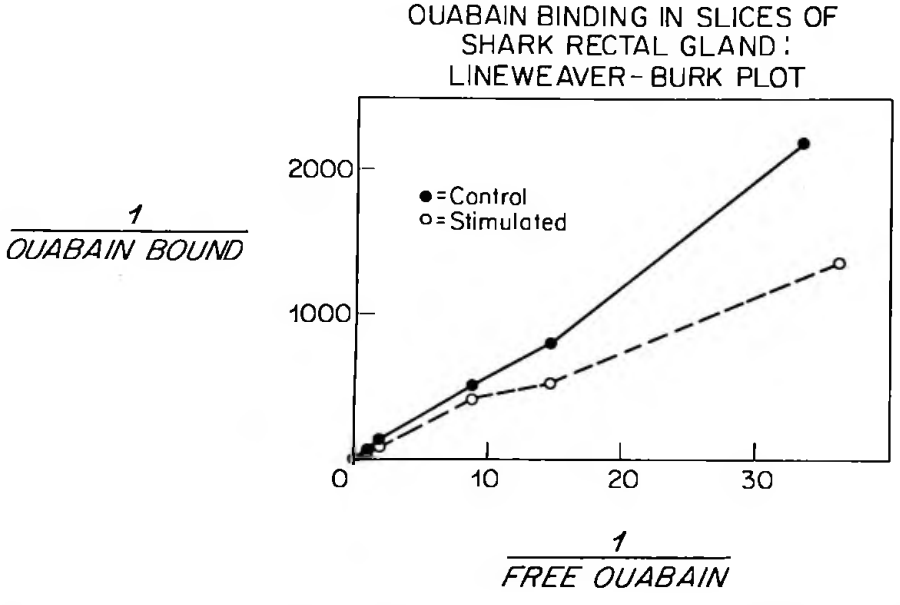

Figure 4.--Lineweaver-Burk plot of the data shown in Figure 3. As the graph indicates, theophylline 10^{-2}M and dibutyryl cyclic AMP 10^{-2}M stimulate ouabain binding by rectal gland slices. However, because of the effect of stimulation reverses at a ouabain concentration of 10^{-6}M the curves cross before the vertical axis; this cannot be seen in this graph because of the scale. Furthermore, both curves are not linear essentially ruling out a single receptor site for ouabain in rectal gland cells.

Lineweaver-Burk and the Scatchard plots effectively rules out a simple binding relation for ouabain to the rectal gland cell suggesting that there is more than one binding site for ouabain in these cells. Calculation of the average affinity, sometimes done in situations like this where the Scatchard plot is curvilinear indicates that cyclic AMP and theophylline increase the affinity for ouabain by 50% from 0.12 1/micromole to 0.18 1/micromole. Models can be developed to fit the experimental data using the law of mass action for analysis of binding.

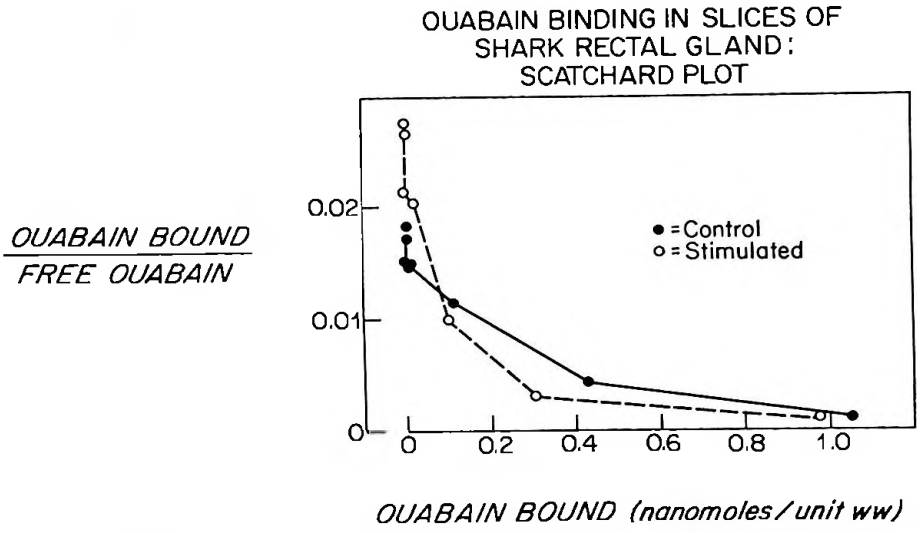

Figure 5.--Scatchard plot of the data shown in Figure 3. The relation between bound ouabain/ free ouabain vs bound ousabin is curvilinear both in the presence and absence of theophylline 10^{-2}M and dibutyryl cyclic AMP 10^{-2}M indicating that there are more than one ouabain binding sites in rectal gland slices. Average affinity calculation indicate that theophylline cyclic AMP increase the affinity for ouabain.

Application of such models to the relation between ouabain bound and ouabain concentration in the incubation medium for the data shown in Figures 3, 4, and 5 suggests that there is more than one receptor site for ouabain in the rectal gland cell and that incubation with cyclic AMP and theophylline changes the affinity for ouabain and possibly the number of receptor sites. Ouabain binding at the high affinity site is increased by cyclic AMP and theophylline.

Mechanism of stimulation of ouabain binding

A plausible mechanism for activation of Na-K-ATPase is that an increase in the movement of sodium into the cell results in a small increase in the intracellular concentration of sodium which would directly activate the

Table I

Effect of stimulation with dibutyryl cyclic AMP (10^{-3}M) and theophylline (10^{-3}M) on ouabain binding by rectal gland slices.

	10^{-9}M ouabain		10^{-8}M ouabain		mean % increase with stimulation
	Basal	Stimulated	Basal	Stimulated	
Control	12.3±0.9(4)	21.6±2.5(4)			78±20(4)**
Furosemide 10^{-4}M	11.8±1.4(4)	24.7±2.9(4)			111±18(4)***
Control	14.3	26.9	154.4±16.4(3)	226.4±20.5(3)	58±11(4)***
Bumetanide 10^{-5}M	15.2	19.8	186.2±14.5(3)	278.3±32.1(3)	46±16(4)*
Control			215.6±14.1(3)	290.3±12.4(3)	37±14(3)*
Litnium chloride (No sodium)			203.0± 9.6(3)	260.1±15.1(3)	29± 9(3)**
Control	14.3	26.9	154.4±16.4(3)	226.4±20.5(3)	58±11(4)***
Sodium nitrate (No chloride)		20.9	181.6±22.8(3)	291.1±37.6(3)	61±11(3)***

Units are picomoles of ouabain bound per gram wet weight. Values are Mean ± SEM(n)
* $p < 0.05$
** $p < 0.025$
*** $P < 0.01$

enzyme. We have evidence in isolated perfused rectal glands, where Na-K-ATPase activity has been inhibited with ouabain, that theophylline and cyclic AMP do increase the rate of sodium and chloride entry into the cell

(Bull. MDIBL 19:70-72, 1979). This increased rate of entry of sodium is blocked by furosemide or bumetanide. High-affinity ouabain binding (at 10^{-9}M and 10^{-8}M) was therefore measured in slices incubated with and without theophylline and cyclic AMP in the presence of furosemide (10^{-4}M) and bumetanide (10^{-5}M). Other experiments were carried out in which sodium was replaced by lithium, or chloride was replaced by nitrate (Table 1) maneuvers that also inhibit secretion by interfering with sodium chloride cotransport. Theophylline and cyclic AMP stimulated ouabain binding even in the presence of furosemide or bumetanide and whether or not sodium or chloride were present in the incubation solution.

From these experiments we conclude that theophylline and cyclic AMP alter the binding of ouabain to rectal gland cells and therefore probably alter the characteristics of membrane Na-K-ATPase. This appears to be the result of an increase in the affinity for ouabain at high-affinity sites on cell membranes although changes in the number of sites cannot be excluded. An increase in secretory activity is not a necessary step for the increase in ouabain binding produced by theophylline and cyclic AMP. Cyclic AMP appears to initiate a series of cellular events that alter membrane Na-K-ATPase directly rather than as a secondary result of ion movements in the course of stimulated secretion.

KINETICS OF ^{35}S-SULFOBROMOPHTHALEIN UPTAKE FROM ALBUMIN SOLUTIONS IN ELASMOBRANCH LIVER

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Recent studies in mammalian systems have suggested that the hepatic uptake of sulfobromophthalein (BSP), bilirubin, and long chain fatty acids may involve a saturable interaction of the albumin with receptor sites on the cell surface (Science 211:1048, 1981). Elasmobranchs offer an opportunity to investigate the nature of these receptor sites, for while they take up and excrete BSP, these marine species lack serum albumin and by inference a specifically evolved albumin receptor.

Uptake kinetics were determined at 15°C by using a multiple steady-state, single-pass perfused liver method as previously described (ibid). Male skates (Raja erinacea, 0.75-1.2 kg) were maintained for up to five days in aerated tanks before livers were isolated by the method of Reed et al., (Am. J. Physiol., in press, 1982). After a 30-min equilibration period during which livers were perfused with recirculating oxygenated Elasmobranch Ringer solution, livers were perfused with a sequence of single-pass test solutions in which either the concentration of ^{35}S-BSP was systematically varied at a fixed albumin concentration, or the concentrations of both ^{35}S-BSP and bovine serum albumin were varied at a fixed (1:50) BSP:albumin molar ratio ($\bar{\nu}$). Uptake was studied at albumin concentrations of 0.05, 0.25 and 0.75% using $\bar{\nu}$ values up to 3; for a fixed $\bar{\nu}$ of 1:50, albumin concentrations were 0.15-1.5%. Five to 6 BSP solutions were each perfused for 3-min in one liver, and effluent samples were obtained for scintillation counting after steady-state uptake was achieved. Each test solution was followed by a 5-min perfusion of a similar albumin solution lacking BSP, which rapidly reduced effluent radioactivity to negligible levels. The net steady-state uptake rate for each solution was calculated as the product of the first-pass extraction fraction, the flow rate per gram of liver and the total BSP concentration. Data were analyzed by non-linear, least-squares computer curve fitting and the results for replicate livers averaged. The equilibrium concentration of free BSP was estimated for each solution by computer analysis using published binding constants (J. Clin. Invest. 45:281, 1966).

Figure 1 relates BSP uptake velocity (solid line) to total BSP concentration over the range 4-112 μM for each of two albumin concentrations. At 0.75% albumin (panel A) saturation of uptake is evident with linear double-reciprocal plots (not shown). The apparent K_m and V_{max} are shown in Table 1. At 0.25% albumin