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Presence of a Sodium-Potassium Chloride Cotransport System in the Rectal Gland of *Squalus acanthias*

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Summary. In order to investigate whether the loop diuretic sensitive, sodium-chloride cotransport system described previously in shark rectal gland is in fact a sodium-potassium chloride cotransport system, plasma membrane vesicles were isolated from rectal glands of *Squalus acanthias* and sodium and rubidium uptake were measured by a rapid filtration technique. In addition, the binding of N-methylfurosemide to the membranes was investigated. Sodium uptake into the vesicles in the presence of a 170 mM KCl gradient was initially about fivefold higher than in the presence of a 170 mM KNO_3 gradient. In the presence of chloride, sodium uptake was inhibited 56% by 0.4 mM bumetanide and 40% by 0.8 mM N-methylfurosemide. When potassium chloride was replaced by choline chloride or lithium chloride, sodium uptake decreased to the values observed in the presence of potassium nitrate. Replacement of potassium chloride by rubidium chloride, however, did not change sodium uptake. Initial rubidium uptake into the membrane vesicles was about 2.5-fold higher in the presence of a 170 mM NaCl gradient than in the presence of a 170 mM $NaNO_3$ gradient. The effect of chloride was completely abolished by 0.4 mM bumetanide. Replacement of the sodium chloride gradient by a lithium chloride gradient decreased rubidium uptake by about 40%; replacement by a choline chloride gradient reduced the uptake even further. Rubidium uptake was also strongly inhibited by potassium. Sodium chloride dependence and bumetanide inhibition of rubidium flux were also found in tracer exchange experiments in the absence of salt gradients. The isolated plasma membranes bound ^{3}[H]-N-methylfurosemide in a dose-dependent manner. In Scatchard plots, one saturable component could be detected with an apparent K_D of 3.5×10^{-6} M and a number of sites n of 104 pmol/mg protein. At 0.8 μM, N-methylfurosemide binding decreased 51% when sodium-free or low-potassium media were used. The same decrease was observed when the chloride concentration was increased from 200 to 600 mM or when 1 mM bumetanide or furosemide were added to the incubation medium. These studies indicate that the sodium-chloride cotransport system described previously in the rectal gland is in fact a sodium-potassium chloride cotransport system. It is postulated that this transport system plays an essential role in the secondary active chloride secretion of the rectal gland.

Key Words sodium-potassium chloride cotransport · chloride transport · "loop diuretics" · rectal gland · furosemide binding sites

Introduction

The rectal gland of the dogfish (*Squalus acanthias*) actively secretes chloride, thereby assisting the animal in maintaining its salt and water balance [1]. Since the organ can be perfused *in vitro* and is composed of a homogenous cell population with extensive infoldings of the basal-lateral plasma membranes it has been used successfully in the past as a model system for biophysical and biochemical studies on active chloride transport [3, 20]. In 1977 Silva et al. proposed a model for active chloride secretion that, based on the sodium dependence and the ouabain sensitivity of the transport, invoked secondary active chloride transport in which a sodium-chloride cotransport system located in the basal-lateral plasma membrane of the transporting cells coupled sodium and chloride movement [20]. Such a system was indeed found in isolated plasma membranes [3]. It could be shown that potassium chloride as compared to potassium nitrate increased sodium uptake into rectal gland plasma membrane vesicles. The uptake studies also demonstrated the sensitivity of this transport system to "loop diuretics" such as furosemide and bumetanide [3, 10].

Recently, in a variety of nonepithelial [5, 17] and epithelial cells [13, 15], a transport system for sodium chloride has been described that is likewise inhibited by loop diuretics but requires for full activity the presence of potassium. It has been postulated to perform sodium-potassium chloride cotransport. Since in the earlier studies with rectal gland plasma membranes potassium was always used as the counter-ion for chloride, it was not possible to deduce from those studies whether the sodium chloride cotransport system found in the plasma membranes actually represented a sodium-

potassium chloride cotransport system. The decrease of chloride secretion observed in the isolated gland when perfused with potassium-free media (F.H. Epstein & P. Silva, *personal communication*) also could not be interpreted unequivocally to demonstrate a potassium dependence of the sodium chloride cotransport system. Chloride secretion depends entirely on the operation of the Na,K-ATPase [20] and this enzyme is inhibited when potassium is removed from the extracellular compartment [7].

We therefore decided to use again isolated plasma membranes to study this problem. Three membrane properties related to the presence of a loop diuretic sensitive, sodium-potassium chloride cotransport system were investigated: potassium dependence of sodium flux, sodium dependence of potassium flux and sodium and potassium dependence of the binding of a diuretic to the transport system. In the experiments described below all of the above-mentioned properties could be demonstrated. The studies therefore provide direct evidence for the presence of a sodium-potassium chloride cotransport system in the basal-lateral plasma membranes of rectal gland cells. It is postulated that this cotransport system is an essential element in the secondary active transport of chloride by the rectal gland.

Materials and Methods

Female dogfish (*Squalus acanthias*) were caught by hook and line in Frenchman's Bay, Maine, during July, 1982. The fish were kept in live cars and used within two days to one week of capture. Isolation of plasma membrane vesicles was carried out at 0 to 4 °C by a modification of the method described by Hokin et al. [6]. The procedure consists of a series of centrifugation steps in which plasma membranes are separated from nuclei, mitochondria and endoplasmic reticulum. The marker enzyme Na,K-ATPase, determined as previously described [11] but without deoxycholate treatment was enriched about fourfold in the final membrane fraction. Protein was determined by the method of Lowry et al. [14]. ^{22}Na uptake and ^{86}Rb uptake were followed using a rapid filtration technique [3]. The binding of ^{3}[H]-N-methylfurosemide was measured in the following way. The reaction was initiated by the addition of 20 µl membranes (100 µg protein) to 65 µl incubation medium. Following a 20 min incubation period a 20-µl sample was removed and pipetted directly in 15 to 20 discrete drops onto a mixed cellulose ester filter (Millipore HAWP, pore size 0.45) kept under suction. The filter was then washed with 3.5 ml ice-cold stop solution (240 mM mannitol, 200 mM KNO_3, 20 mM Tris-HEPES, 1.2 mM $Mg(NO_3)_2$, pH 7.6); the washing took less than 2 sec. Experiments were performed at 15 °C in duplicate for transport and in triplicate for binding. The composition of the incubation media is given in the legends to Figures and Tables. The membranes were usually suspended in 200 mM mannitol, 20 mM Tris-HEPES, 1.2 mM $Mg(NO_3)_2$, pH 7.6. The filters were transferred into liquid scintillation fluid (Econofluor, New England Nuclear, Boston, Mass.) and counted by standard liquid scintillation techniques. The values were corrected for the amount of radioactivity bound to the filters in the absence of plasma membranes and expressed as picomoles by using the specific activity determined separately for each incubation medium. ^{22}Na (carrier free) and ^{86}RbCl (0.06 to 4.2 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. [^{3}H]-N-methylfurosemide and N-methylfurosemide were gifts from Hoechst A.G., Frankfurt am Main, West Germany. The purity of the compound was >99% and was checked routinely by thin-layer chromatography. All other chemicals were of the highest grade of purity commercially available.

Results

Three functional parameters of the putative loop diuretic sensitive sodium-potassium chloride cotransport system will be analyzed in the following sections. First the effect of potassium on the chloride-dependent and bumetanide-sensitive sodium uptake by the membrane vesicles will be investigated. Then the potassium flux properties of the membranes – using rubidium as potassium substitute – will be discussed with respect to chloride dependence, sodium dependence and the action of bumetanide. Thirdly, the binding of one loop diuretic to the transport system will be used to demonstrate the interactions of sodium, potassium and chloride with the transport system.

I. Sodium Uptake by Rectal Gland Plasma Membranes

General Characteristics. As demonstrated previously [3], sodium uptake into rectal gland plasma membrane vesicles is markedly stimulated in the presence of a potassium chloride gradient. This phenomenon is shown in Fig. 1. During the first 2 min of incubation, sodium uptake in the presence of KCl is about 5 times higher than in the presence of KNO_3. After 2.5 min of incubation in the chloride medium the amount of sodium present in the vesicles is about 35% higher ($P<0.05$) than the amount present after 120 min of incubation. This finding indicates that sodium is transiently accumulated (overshoot) inside the vesicle, probably driven by the KCl gradient. In the presence of the loop diuretic bumetanide, the initial uptake rate in the presence of KCl is lower and no overshoot is observed. Taken together, the data in Fig. 1 show that sodium movement across the rectal gland plasma membrane is to a large extent chloride gradient dependent and that this chloride-dependent sodium flux can be inhibited (although not completely) by the loop diuretic bumetanide.

N-methylfurosemide, the radioactively labeled furosemide analog used in the binding studies de-

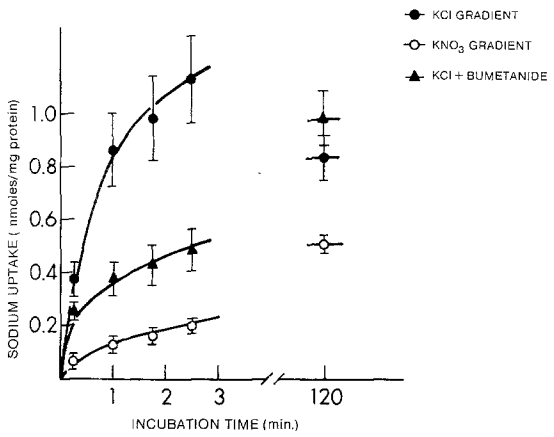


Fig. 1. Sodium uptake by rectal gland plasma membrane vesicles: Effect of chloride and bumetanide. Vesicles containing 200 mM mannitol, 20 mM Tris-HEPES (pH 7.6), and 1 mM $Mg(NO_3)_2$ were incubated in a medium containing in addition 0.4 mM $NaNO_3$ and 170 mM KCl or 170 mM KNO_3. (●–●) uptake in the presence of the KCl gradient, (o–o) uptake in the presence of the KNO_3 gradient, (▲–▲) uptake in the presence of KCl and 0.4 mM bumetanide. The results represent mean values ± SEM derived from 8 experiments

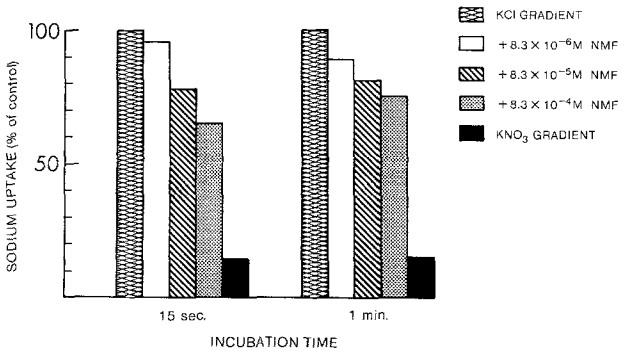


Fig. 2. Sodium uptake by rectal gland plasma membrane vesicles: Effect of N-methylfurosemide. Sodium uptake was determined as detailed in the legend to Fig. 1 in the presence of a KCl gradient, a KNO_3 gradient and increasing amounts of N-methylfurosemide. Mean values derived from 2 experiments are given

scribed below, also inhibits chloride-dependent sodium uptake (Fig. 2). At the highest concentration tested (0.83 mM) the inhibition was about 40%.

B. Effect of Potassium Removal on Chloride-Dependent Uptake. In order to determine whether potassium was necessary for chloride stimulation of the sodium flux, potassium was replaced by lithium chloride or choline chloride. The results of these experiments are compiled in Table 1. In the first series of experiments potassium was replaced by rubidium chloride or by lithium chloride. No significant changes in sodium uptake are observed when rubidium replaces potassium. In the

Table 1. Effect of potassium removal on sodium uptake by rectal gland plasma membranes

Incubation medium		Sodium uptake after:			*n*
		15 sec	1 min	1 min 45 sec	
Series 1					
KCl	(170 mM)	39.9 ± 8.9	90.5 ± 23.7	118 ± 32.3	2
RbCl	(170 mM)	32.8 ± 4.6	79.2 ± 11	107 ± 16.6	2
LiCl	(170 mM)	11.6 ± 7.2	11.1 ± 0.8	16.9 ± 0.15	2
Series 2					
KCl	Choline chloride				
170	0	22.8 ± 3.5	56.0 ± 6.8	73.7 ± 10.9	3
50	120	19.4 ± 3.5	45.1 ± 10.9	64.4 ± 15.6	3
20	150	10.9 ± 0.8	24.2 ± 1.7	36.4 ± 3.9	3
10	160	9.7 ± 0.7	21.4 ± 1.8	27.8 ± 2.3	3
0	170	7.0 ± 0.5	13.3 ± 1.9	19.7 ± 2.9	3

Vesicles containing 200 mM mannitol, 20 mM Tris-HEPES (pH 7.6) and 1 mM $Mg(NO_3)_2$ were incubated in a medium containing, in addition, 0.4 mM $NaNO_3$ and 170 mM salt as shown above.

The values are expressed as percent of the uptake observed for the different incubation media after 120 min of incubation, they represent mean values ± SEM derived from *n* experiments. The data obtained in paired experiments are depicted in series 1 and series 2.

presence of lithium chloride, however, sodium uptake is drastically reduced. In view of a possible direct inhibitory effect of lithium on sodium uptake via competition for the sodium binding site of the transport molecule, a second series of experiments was conducted in which potassium chloride was replaced by choline chloride in a stepwise fashion. As evident from Table 1, a decrease in potassium concentration was accompanied by a decrease in sodium uptake. A 50% reduction was observed between 50 and 20 mM KCl. These results clearly indicate that potassium, in addition to chloride, stimulates sodium transport – one property to be expected when a sodium-potassium chloride cotransport system is present in the membranes.

II. Rubidium Uptake into Rectal Gland Plasma Membranes

A. Effect of Sodium, Chloride and Bumetanide under Gradient Conditions. As demonstrated above, rubidium chloride stimulates sodium uptake into rectal gland plasma membrane vesicles. If this stimulation is due to the operation of a sodium-potassium chloride cotransport system it should be possible to demonstrate a stimulation of rubidium flux by sodium and chloride. Experiments addressing this question are shown in Fig. 3.

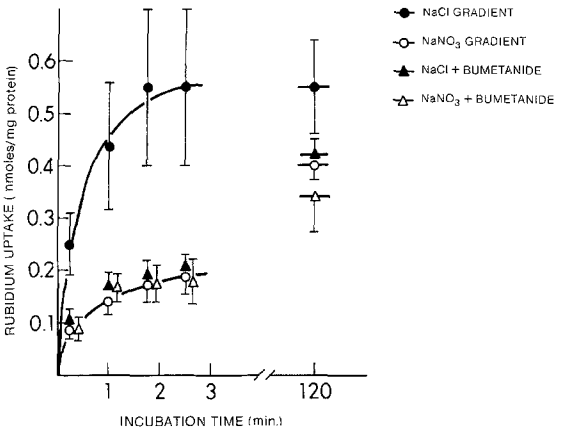


Fig. 3. Rubidium uptake by rectal gland plasma membrane vesicles: Effect of chloride and bumetanide. Vesicles containing 200 mM mannitol, 20 mM Tris-HEPES (pH 7.6) and 1 mM $Mg(NO_3)_2$ were incubated in a medium containing, in addition, 0.4 mM $RbNO_3$ and 170 mM NaCl or 170 mM $NaNO_3$. (●–●) uptake in the presence of the NaCl gradient, (○–○) uptake in the presence of the $NaNO_3$ gradient, (▲–▲) uptake in the presence of NaCl and 0.4 mM bumetanide, (△–△) uptake in the presence of $NaNO_3$ and 0.4 mM bumetanide. The results represent mean values ±SEM derived from 3 experiments

Table 2. Effect of potassium on rubidium uptake by rectal gland plasma membranes

Incubation medium	Rubidium uptake after:		
	15 sec	1 min	120 min
NaCl	44.2	77.6	100
NaCl + 10 mM K^+	19.5	31.8	70.7
$NaNO_3$	14.7	25.6	74.4
$NaNO_3$ + 10 mM K^+	8.8	15.9	59.4

The values are expressed as percent of the equilibrium value obtained in NaCl medium (0.552 pmol/mg protein) and represent mean values derived from three experiments.

In the presence of a sodium chloride gradient, rubidium is taken up very rapidly by the vesicles and reaches equilibrium after 3 min of incubation. In the presence of sodium nitrate, rubidium uptake is markedly reduced. In the early phase of incubation, rubidium uptake in sodium chloride medium is 2.5-fold higher than in sodium nitrate. Bumetanide at 0.4 mM inhibits the chloride-dependent rubidium uptake completely; in the absence of chloride, bumetanide has no effect. Rubidium uptake after 120 min of incubation is 0.3 nmol in the case of sodium nitrate and 0.5 nmol in the presence of sodium chloride. The difference is only marginally significant and cannot be explained, at the moment.

In order to establish whether rubidium uptake was indeed indicative of potassium movements, potassium was added to the incubation medium (Table 2). In the presence of 0.4 mM rubidium and a sodium chloride gradient 10 mM KCl reduces rubidium uptake to a level similar to that observed in the presence of sodium nitrate. Consistent with this observation, potassium nitrate decreases rubidium uptake in the presence of sodium nitrate. These results establish that rubidium acts as a substitute for potassium in this transport system. They also demonstrate that rubidium uptake, similar to sodium uptake, is stimulated by a chloride gradient and is inhibited by the loop diuretic bumetanide. If the rubidium flux would occur via the sodium-potassium chloride cotransport system, removal of sodium should inhibit rubidium uptake. Results of sodium replacement experiments are given in Table 3. When sodium is replaced by lithium, the uptake of rubidium is inhibitied 53%; when sodium is replaced by choline, rubidium uptake decreases by almost 80%. Stepwise addition of sodium leads to a concomitant increase in rubidium

Table 3. Effect of sodium removal on rubidium uptake by rectal gland plasma membranes

Incubation medium	Rubidium uptake after:				*n*
	15 sec	1 min	1 min 45 sec	120 min	
NaCl (170 mM)	44.2±7.0	77.6 ±13	96.2±14.3	100	3
LiCl (170 mM)	23.9±6.2	45.25±11.4	59.6±15.5	100	3
Choline chloride (170 mM)	9.6	18.0	29.5	100	1
160 mM + 10 mM Na	10.9	20.9	27.4	100	1
150 mM + 20 mM Na	17.9	32.4	51.2	100	1
110 mM + 50 mM Na	22.8	49.9	69.2	100	1

Values are given in % of the equilibrium value observed for each experimental set up and represent mean values ±SEM derived from *n* experiments.

Table 4. Effect of sodium, chloride and bumetanide on rubidium exchange

Incubation medium	Exchange rate	r^2
NaCl	0.37 ± 0.04	0.94
NaCl + bumetanide[a]	0.16 ± 0.03	0.90
$NaNO_3$[a, b]	0.16 ± 0.01	0.90
$NaNO_3$ + bumetanide[a, b]	0.13 ± 0.02	0.90
Choline chloride[a]	0.25 ± 0.03	0.98
Choline chloride + bumetanide[a, b]	0.20 ± 0.03	1.00

[a] Significantly different ($P < 0.05$) from NaCl medium.
[b] Not significantly different from exchange in the same medium without bumetanide.

The results represent mean values ± SEM derived from 3 experiments. Bumetanide was used in a final concentration of 0.4 mM. The exchange rate was calculated from a plot of $\ln(c_\infty - c_t/c_\infty)$ versus the incubation time, c_∞ = intravesicular concentration of tracer after 120 min, c_t tracer concentration after 15 sec, 1 min, 1 min 45 sec and 2 min 30 sec, respectively. r^2 indicates the probability for the regression line calculated to obtain the value for the exchange rate.

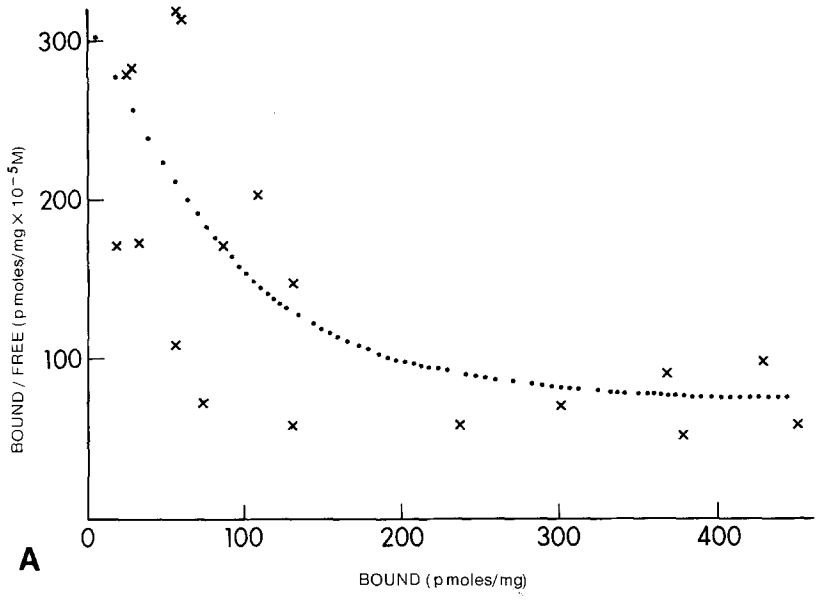
uptake. Although the latter results were obtained only in one experiment the use of various time points and sodium concentrations supports the significance of the findings. Thus rubidium flux across the vesicle membrane is stimulated by sodium and chloride and is inhibited by bumetanide, a picture quite complementary to the dependence of sodium uptake on potassium and chloride and its inhibition by bumetanide.

B. Effect of Sodium, Chloride and Bumetanide under Tracer Exchange Conditions. Since the experiments mentioned above were performed under ion gradient conditions where the electrical potential difference across the membranes may change when the salt gradients are altered, the effects of sodium and chloride on rubidium flux could be due to electrical coupling phenomena rather than flux coupling via a cotransport system. In order to minimize such interference, tracer exchange studies in the absence of salt gradients were performed. The results of these studies are compiled in Table 4. They essentially corroborate the findings made under gradient conditions. In the sodium chloride medium the rate of rubidium uptake is about 2.3-fold higher than in the sodium nitrate medium and bumetanide blocks the effect of sodium chloride completely. Replacement of sodium by choline decreases the rubidium uptake rate by 30% and the effect of bumetanide on the rubidium uptake is blunted. The tracer exchange experiments demonstrate directly that flux coupling between sodium, potassium and chloride takes place via a cotransport system that is inhibited by the loop diuretic bumetanide.

III. Binding of N-Methylfurosemide to Rectal Gland Plasma Membranes

A. General Characteristics. The inhibition by loop diuretics of the postulated sodium-potassium chloride cotransport system requires a specific interaction between the transporter and the inhibitor. It should therefore be possible – in analogy to phlorizin and the sodium-D-glucose cotransport system [8] – to use the binding of the inhibitor as an indicator for the presence of sodium, potassium and chloride binding sites at the transporter molecule. As demonstrated above, N-methylfurosemide inhibits potassium chloride-dependent sodium uptake and thus can be assumed to interact with the sodium-potassium chloride cotransport system. Indeed, when binding of [^{3}H]-N-methylfurosemide (NMF) to rectal gland plasma membranes is investigated, a concentration-dependent association of NMF with the membranes is observed. When the results are analyzed according to Scatchard (Fig. 4*A*) a nonlinear plot is obtained that can be resolved in the first approximation into two components. One component is directly proportional to the concentration of NMF used in the incubation medium while the other demonstrates saturation kinetics. By extrapolation of values obtained for binding of NMF observed at high concentrations a straight line is obtained that can be described by the equation $b = 8.5 \times 10^{-6} f$, where b stands for the amount bound per mg protein and f for the free NMF concentration. When the three experiments are plotted individually following graphical subtraction of the linear component $b = 8.5 \times 10^{-6} f$, linear Scatchard plots are obtained (Fig. 4*B*) from which an apparent K_D of $3.5 \pm 1.2 \times 10^{-6}$ M (mean value ± SD) and a number of binding sites of 104 ± 21 pmol/mg protein can be derived. The dotted line in Fig. 4*A* represents the calculated sum of the two components. At 0.8×10^{-6} M – the concentration used in the experiments described next – total binding of NMF is 28.1 pmol, 60 to 70% of which represents binding to the saturable component.

B. Effect of Bumetanide and Furosemide on NMF Binding. In order to verify our assumption that NMF binding occurs to a binding site for loop diuretics, the effect of bumetanide and furosemide on NMF binding was investigated. At a concentration of 0.8×10^{-6} M, NMF binding decreases to $44.5 \pm 5.5\%$ ($n = 3$) of the control in the



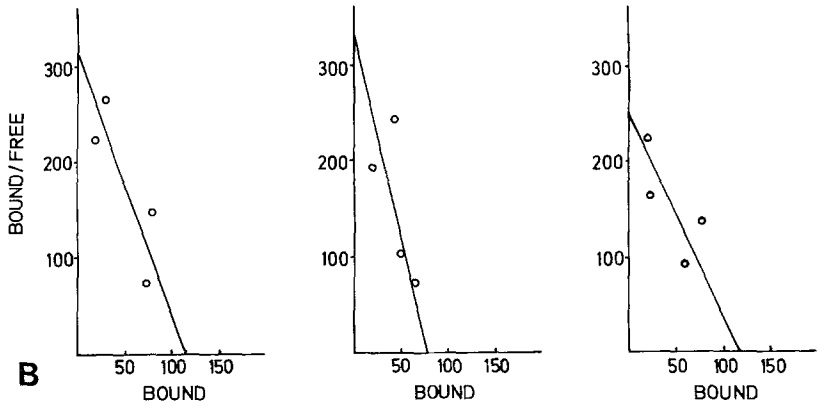


Fig. 4. Binding of N-methylfurosemide to rectal gland plasma membrane vesicles: concentration dependence. In *A* the results obtained in all experiments are represented in a Scatchard plot. The dotted line was calculated assuming the presence of one saturable component ("K_D" 3.5×10^{-6} M, $n = 104$ pmol/mg) and one nonsaturable component ($b = 8.5 \times 10^{-6} f$). In *B* Scatchard analysis following subtraction of the linear nonsaturable component is demonstrated for three independent determinations of N-methylfurosemide binding

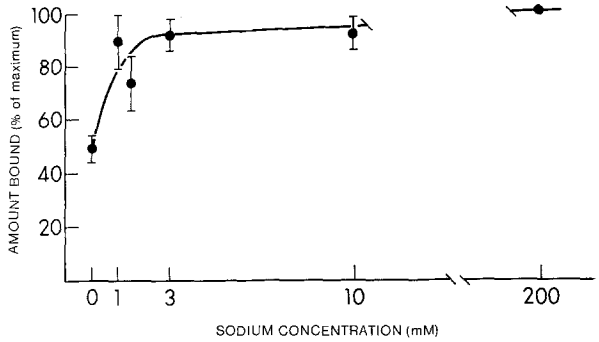


Fig. 5. Binding of N-methylfurosemide to rectal gland plasma membrane vesicles: effect of sodium. Plasma membranes were incubated for 20 min with 0.8×10^{-6} M NMF in 100 mM mannitol, 20 mM KCl, 1.2 mM $Mg(NO_3)_2$, 20 mM Tris-HEPES (pH 7.4) and increasing concentrations of sodium chloride; choline chloride was used as cation replacement. The amount of NMF bound to the membranes was determined by a rapid filtration technique. The results represent mean values ±SEM derived from 3 experiments and are given as percent of the binding observed at 200 mM NaCl and zero choline chloride

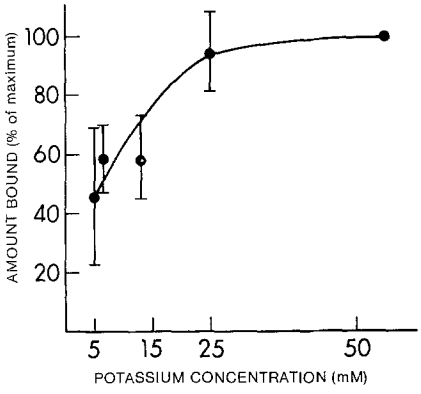


Fig. 6. Binding of N-methylfurosemide to rectal gland plasma membrane vesicles: effect of potassium. Plasma membranes were incubated for 20 min with 0.8×10^{-6} M NMF in 100 mM mannitol, 200 mM NaCl, 1.2 mM $Mg(NO_3)_2$, 20 mM Tris-HEPES (pH 7.4) and decreasing amounts of KCl; NaCl was used as cation replacement. The amount of NMF bound to the membranes was determined by a rapid filtration technique. The results represent mean values ±SEM derived from 3 experiments and are given as percent of the binding observed at 50 mM KCl

Table 5. Effect of anions on the binding of [^{3}H]-N-methylfurosemide to rectal gland plasma membranes

	% control	*n*
400 mM Cl	47±14	3
400 mM NO_3	61±8	2
400 mM gluconate	110	1

The control incubation medium contained 200 mM NaCl, 10 mM KCl, 20 mM Tris-HEPES and 1.2 mM $Mg(NO_3)_2$. Sodium salts of the anions were used. The results represent mean values of *n* experiments with the standard error of the mean values.

presence of 10^{-3} M bumetanide and to 23.3±10% ($n=3$) in the presence of furosemide. Thus at least 56% of NMF bound to the membranes share its binding site with the two other loop diuretics.

C. Effect of Sodium, Potassium and Chloride on NMF Binding. In Figs. 5 and 6 experiments are compiled in which the ionic composition of the incubation medium was altered to see whether any of the ions transported by the putative sodium-potassium chloride cotransport system affected the binding of NMF to the transport system. In Fig. 5 the sodium concentration of the incubation was lowered from 200 mM – the physiological level for the shark rectal gland – to zero. This maneuver results in a decrease in binding of NMF by 56%. Interestingly enough the sodium dependence of NMF binding becomes evident already at very low sodium concentrations and a half-maximum stimulation of NMF binding is observed at about 0.5 mM sodium. The scatter of the experiments precludes a precise determination of the sodium affinity of the binding site at the present time.

In Fig. 6 the effect of potassium on NMF binding was investigated. In the range between 50 and 5 mM, the lowest potassium concentration tested, a decrease in potassium concentration leads to a significant decrease in binding. The binding at 5 mM potassium amounts to 46±2% ($n=4$) of the binding observed at 50 mM potassium.

The effect of chloride on NMF binding is depicted in Table 5. In this series of experiments the chloride concentration was increased from 210 to 600 mM. This was done following the reasoning that part of the NMF molecule might interact directly with the chloride binding site(s) of the transport system. Indeed, it was observed that an increase in chloride concentration decreases NMF binding to about the same level observed in sodium-free or low-potassium medium. Addition of 400 mM nitrate instead of chloride also decreased the binding but to a lesser extent; gluconate had no effect on NMF binding.

Discussion

In the experiments described above three different properties of rectal gland plasma membranes have been studied: sodium uptake, rubidium uptake and binding of the loop diuretic N-methylfurosemide. In the following, these three properties will first be discussed separately and then the significance of the results with regard to the putative sodium-potassium chloride cotransport system will be considered.

The sodium uptake studies represent an extension of our earlier work in which an effect of chloride and furosemide on sodium uptake was observed [3]. In the current studies a much larger stimulation of sodium uptake by chloride was found and sodium uptake showed a slight but significant overshoot indicating a transient accumulation of sodium inside the membrane vesicles. Since the uptake of sodium in the absence of chloride was similar in both studies, the most probable cause of the higher stimulation and the overshoot is the increase in the potassium chloride from 100 (previous studies) to 170 mM (current studies), which exerts a larger driving force for sodium uptake.

The driving force is probably exerted both by the chloride and by the potassium ion as evidenced by the potassium replacement experiments. These experiments were performed using two different ions to replace potassium, lithium and choline, because at such high concentrations both substitutes can exert unspecific effects on the transport. Lithium is known to share transport sites with sodium in sodium cotransport systems [12] and with a sodium concentration of 0.4 mM, 170 mM Li can compete with sodium effectively even if the affinities of the transport system for the two ions differ by two orders of magnitude. That such a situation might indeed exist can be deduced from the higher rate of rubidium uptake observed when sodium was replaced by lithium rather than by choline. Choline chloride itself does not seem to affect the transport system directly since an increase from zero to 120 mM (Table 1) decreases sodium transport insignificantly. The further stepwise reduction of potassium with only small additional increase of choline leads, however, to a marked reduction in sodium flux. Thus, especially with reference to the choline experiments it seems quite clear that potassium enhances sodium movement across the

membrane. The potassium effect can also be elicited by rubidium, thus potassium and rubidium seem to share a common site of action.

The experiments reported above on the inhibition of sodium transport by bumetanide and N-methylfurosemide provide evidence that both substances have a similar site of action as furosemide, whose inhibitory action on sodium transport in the vesicles has been reported previously [3, 10]. The inability of 0.8 mM N-methylfurosemide to completely inhibit chloride-dependent sodium uptake may be due to the low concentration of sodium (0.4 mM) used in the incubation medium. As has just been described, the binding of N-methylfurosemide is dependent on the presence of sodium; thus in the presence of a low sodium concentration, incomplete inhibition of sodium uptake or lower affinity of the diuretic might be expected. The same reasoning may hold for the incomplete inhibition of sodium flux (at 0.4 mM Na) by bumetanide as contrasted to the complete inhibition of rubidium flux in the presence of 170 mM Na. Despite these difficulties caused by the experimental set up the results obtained for bumetanide corroborate findings in the intact gland where chloride secretion was inhibited both by furosemide and bumetanide [9, 18, 20]. It can therefore be concluded that the isolated membranes at least qualitatively reflect the properties of the membranes *in vivo*.

With respect to rubidium uptake by the rectal gland plasma membranes the question has to be raised whether the uptake by the plasma membranes represents binding to the membranes and/or uptake into the intravesicular space. This question can be answered by comparing the apparent intravesicular space for rubidium and sodium that can be calculated from the uptake of the isotopes after long-term incubation. For rubidium, the intravesicular space in sodium chloride medium amounts to 1.3 µl/mg protein, the corresponding value for sodium is 2.3 µl/mg protein. The lower value for rubidium indicates that there is no binding of rubidium to the membranes but rather that some rubidium might be lost from the intravesicular space during the washing procedures. Part of the difference can also be attributed to the fact that rubidium and sodium uptake were investigated in different membrane preparations. It is known that a variation of the intravesicular space by a factor of two is quite common in such preparations.

That rubidium uptake in the sodium chloride medium occurs mainly via a transport system and not only by simple diffusion is evident from the inhibition of the uptake by potassium and bumetanide and from its stimulation by chloride and sodium. The inhibition by potassium can be explained by assuming that potassium is also a substrate of the transport system and therefore competes with rubidium for its binding site. Stimulation of the uptake by sodium and chloride can either be due to a direct stimulatory effect on the transport system or due to the generation of diffusion potentials across the membrane. This potential can be expected to be vesicle inside negative in both instances because in the choline chloride medium, sodium has been replaced by a less permeant cation and in the sodium nitrate medium, chloride has been replaced by a more permeant anion. Since, however, all the above-mentioned effects are observed also under tracer exchange conditions in the absence of salt gradients, it can be concluded that the stimulation of rubidium uptake by sodium and chloride is caused by a direct interaction of the two ions with the rubidium transport system. The results therefore demonstrate that the plasma membranes of the rectal gland contain a transport system for rubidium that can be shared by potassium, is stimulated by chloride, is inhibited by bumetanide and is to a large extent sodium dependent. Rubidium uptake in the absence of chloride is bumetanide insensitive but is inhibited significantly by potassium. These observations might indicate that another pathway (channel?) for rubidium and potassium transfer is present in the rectal gland plasma membranes, the nature of which remains to be elucidated.

With respect to the interaction of N-methylfurosemide with the membranes, the following points are noteworthy. The association of the ligand with the membranes is probably a combination of binding to the membranes, partition into the membrane and/or uptake into the intravesicular space present in the membrane fraction. Binding to the membranes should follow a Langmuir adsorption isotherm with a defined number of binding sites and a given dissociation constant. Partition into the membrane and uptake into the vesicles, when determined at equilibrium, would be a linear function of the free concentration of the ligand. The linear component described as $b = 8.5 \times 10^{-6}$ M $\times f$ probably represents partition of the ligand into the membranes because it can be calculated that the intravesicular space (~ 1 µl) contributes only about 13% to the linear component. The binding component obtained can be fitted in the Scatchard plot by linear regression and thus probably represents one class of binding sites. The maximum number of sites of 104 pmol/mg protein is probab-

ly underestimated to a certain extent. With dissociation constant of 3.5×10^{-6} M some dissociation might occur during the removal of the free ligand by rapid filtration. Forbush and Palfrey [4] obtained a number of 200 nmol/mg protein with a similar method but with a ligand that has a higher affinity to the transport system. This thousandfold difference cannot be attributed completely to methodological artifact; other factors such as purity of the membrane fraction and correction for partition into the membrane and/or the intravesicular uptake might be more important.

The apparent affinity of the transport system for N-methylfurosemide obtained in the binding experiments, i.e. in the presence of 200 mM NaCl and 10 mM KCl, is quite close to the apparent affinity of the parent compound furosemide in the intact gland [18] where similar salt concentrations are used in the perfusion studies. The affinity for NMF that can be estimated in the sodium uptake studies with isolated membranes is almost two orders of magnitude lower. This discrepancy can be explained by the sodium dependency of the binding of NMF. In the transport studies only 0.4 mM Na are present compared to 200 mM in the binding studies. The contact time between the transport system and the inhibitor might also be of importance. Preliminary studies on the time dependence of NMF binding indicate that maximum binding is achieved after about 2 min of incubation. In the transport studies the membranes were not preincubated with NMF, the actual contact time was therefore less than the time required for optimal binding.

The binding sites for NMF also accept furosemide and bumetanide. Although the affinity of the binding sites for these diuretics has not yet been determined, the inhibition observed supports the assumption that the NMF binding sites represent binding sites for loop diuretics in general and therefore can be considered a part of the transport system under investigation. This assumption is supported by the fact that sodium and potassium, ions whose transport is inhibited by loop diuretics, influence the binding of NMF to these sites.

It might be expected that the binding of N-methylfurosemide at 0.8 μM should be inhibited equally by 1 mM furosemide or 1 mM bumetanide. However, the unspecific interaction of the membrane with NMF might be more greatly affected by furosemide than bumetanide due to structural identity in the hydrophobic region of the molecule, thus explaining the increased inhibition of NMF binding seen with furosemide in contrast to bumetanide.

The stimulation of NMF binding by sodium has an analogy in the stimulation of phlorizin binding to the sodium-D-glucose cotransport system in renal and intestinal brush border membranes [8]. It is generally assumed that this cotransport system possesses a sodium binding site and a glucose binding site, the affinity of the glucose binding site being enhanced by the occupancy of the sodium site. Since phlorizin binds to the glucose binding site its affinity is also increased in the presence of sodium [8]. If this model is applied to the binding site for NMF, a sodium binding site could be envisaged whose occupancy increases the affinity of the binding site for NMF. The effect of potassium on NMF binding could be explained in the same way by postulating that the transport system has not only a sodium binding site but also a potassium binding site. The two are not identical because the stimulation of binding by potassium is observed at a sodium concentration that completely saturates the sodium site. In extending the analogy to the sodium-D-glucose cotransport system we also can offer an explanation for the effect of chloride on NMF binding. Since chloride is the ion that is actually transported actively across the gland epithelium it can be considered as the primary substrate for the transport system whereas sodium and potassium, are the cotransported ion species. The transport system therefore definitely must have a chloride binding site. We postulate that NMF and perhaps other loop diuretics bind to the chloride binding sites and that chloride competes for this binding. This could well be the explanation for the inhibition of NMF binding by high chloride concentrations. Such high chloride concentrations had to be used because the affinity of the transport system for NMF with a K_D of 3.5×10^{-6} M is much higher than the chloride affinity of about 50 mM observed in perfusion studies in the intact gland [20]. If these constants are used to calculate the ratio of binding sites occupied by chloride and those occupied by NMF a value of 10/1 is obtained for 600 mM chloride and 0.8×10^{-6} M NMF. Under the given experimental conditions therefore chloride can effectively compete with NMF for the binding. Clearly, further evidence for this model has to be established in experiments where the interaction of all ions studied so far with the binding site has to be studied systematically. The limited amount of radioactivity available precluded such experiments in the current investigation.

In Fig. 7 a scheme is presented that summarizes the results obtained in the sodium flux experiments, the rubidium flux experiments and the

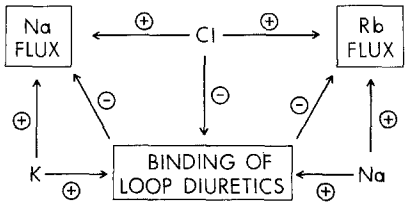


Fig. 7. Synopsis of the results obtained in this study, the interaction of sodium, rubidium (potassium), chloride and loop diuretics is indicated by arrows. ⊕ indicates stimulation of a process; ⊖ indicates inhibition of a process

NMF binding studies. Sodium flux was inhibited by loop diuretics and stimulated by chloride. Rubidium flux was likewise inhibited by loop diuretics and stimulated by chloride. Sodium in turn stimulated rubidium flux in the same manner as rubidium stimulated sodium flux. NMF binding was stimulated by both sodium and potassium but inhibited by chloride. A transport system that could explain all the results described is a sodium-potassium chloride cotransport system whose chloride site interacts with the loop diuretics.

It is interesting to note that transport systems with similar properties have already been described in avian [17] and human red blood cells [2], in Ehrlich's ascites tumor cells [5], in cultured kidney cells [15] and most recently in the thick ascending limb of Henle's loop in rabbit kidney [13]. The latter observation is especially noteworthy since the thick ascending limb of Henle's loop also performs active chloride transport. It has been postulated that the transport system in the TALH cells facilitates the intracellular accumulation of chloride across the luminal membrane and renders the active chloride transport secondary active [13].

A similar model can now be proposed for the rectal gland. Since the plasma membranes studied in our experiments are primarily derived from the contraluminal cell face, and since the loop diuretics also act at this cell side in the intact gland, it can be concluded that the sodium-potassium chloride cotransport system is localized in the contraluminal plasma membrane. Transepithelial chloride secretion could thus be envisaged to first involve accumulation of chloride inside the cell against its electrochemical potential by the action of the sodium-potassium chloride cotransport system. The exit step across the luminal membrane would be sodium independent and governed by the electrochemical potential difference for chloride across this membrane. Potassium would recycle across the contraluminal membrane and might also be involved in the chloride exit across the luminal membrane. Preliminary experiments using barium have already provided some evidence for a recycling process. Perfusion of the rectal gland with barium-containing solutions inhibited chloride secretion probably by blocking the potassium channel through which recycling occurs [19].

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References

1. Burger, J.W., Hess, W.N. 1960. Function of the rectal gland in spiny dogfish. *Science* **131**:670–671
2. Chipperfeld, A.R. 1980. An effect of chloride on (Na+K) cotransport in human red blood cells. *Nature (London)* **286**:281–282
3. Eveloff, J., Kinne, R., Kinne-Saffran, E., Murer, H., Silva, P., Epstein, F.H., Stoff, J., Kinter, W.B. 1978. Coupled sodium and chloride transport into plasma membrane vesicles prepared from dogfish rectal gland. *Pfluegers Arch.* **378**:87–92
4. Forbush, B., Palfrey, H.C. 1982. Bumetanide and benzmetanide binding to membranes from shark rectal gland and canine kidney. *Biophys. J.* **37**:161 a
5. Geck, P., Pietrzyk, C., Burckhardt, B.C., Pfeiffer, B., Heinz, E. 1980. Electrically silent cotransport of Na^+, K^+, and Cl^- in Ehrlich cells. *Biochim. Biophys. Acta* **600**:432–447
6. Hokin, L.E., Dahl, J.L., Deupree, J.D., Dixon, J.F., Hackney, J.F., Perdue, F. 1973. Studies on the characterization of the sodium-potassium transport adenosine triphosphatase. *J. Biol. Chem.* **248**:2593–2605
7. Joergensen, P.L. 1980. Sodium and potassium ion pump in kidney tubules. *Physiol. Rev.* **60**:864–917
8. Kinne, R. 1976. Properties of the glucose transport system in renal brush border membrane. *Curr. Top. Memb. Trans.* F. Bronner and A. Kleinzeller, editors. pp. 209–267. Academic Press, New York
9. Kinne, R., Kinne-Saffran, E. 1979. Effect of loop diuretics on the salt secretion in shark rectal gland. *Mar. Ecol.* **1**:129–132
10. Kinne, R., Kinne-Saffran, E., Epstein, J., Silva, P., Epstein, F.H., Miller, D., Eveloff, J. 1979. Effect of "loop diuretics" on sodium transport by plasma membrane vesicles isolated from rectal gland of *Squalus acanthias*. *Bull. Mt. Desert Isl. Biol. Lab.* **19**:92–95
11. Kinne, R., Schmitz, J.E., Kinne-Saffran, E. 1971. The localization of Na–K-ATPase in the cells of rat kidney cortex. A study on isolated plasma membranes. *Pfluegers Arch.* **329**:191–206
12. Kinsella, J.L., Aronson, P.S. 1980. Properties of the Na^+–H^+ exchanger in renal microvillus membrane vesicles. *Am. J. Physiol.* **238**:F461–F469

13. Koenig, B., Ricapito, S., Kinne, R. 1983. Na−K−Cl cotransport in plasma membrane vesicles isolated from thick ascending limb of Henle's loop. *Pluegers Arch. (submitted)*
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275
15. McRoberts, J.A., Erlinger, S., Rindler, M.J., Saier, M.H. 1982. Furosemide sensitive salt transport in Madin-Darby canine kidney cell line. Evidence for cotransport of Na, K, and Cl. *J. Biol. Chem.* **257**:2260–2266
16. Musch, M., Field, M., Frizzell, R.A. 1981. Active K^+ transport by the intestine of the flounder *Pleuronectes americanus:* Evidence for cotransport with Na and Cl. *Bull. Mt. Desert Isl. Biol. Lab.* **21**:95–99
17. Palfrey, H.C., Feit, P.W., Greengard, P. 1980. cAMP stimulated cation cotransport in avian erythrocytes: Inhibition by "loop" diuretics. *Am. J. Physiol.* **238**:C139–C148
18. Palfrey, H.C., Silva, P., Epstein, F.H. 1980. Effects of ethacrynic acid and related compounds on active Cl secretion by the dogfish rectal gland. *Bull. Mt. Desert Is. Biol. Lab.* **20**:34–36
19. Silva, P., Epstein, J., Myers, M., Stevens, A., Epstein, F.H. 1981. Inhibition of chloride secretion by $BaCl_2$ in the rectal gland of the spiny dogfish *Squalus acanthias. Bull. Mt. Desert Isl. Biol. Lab.* **21**:12–13
20. Silva, P., Stoff, J., Field, M., Fine, L., Forrest, J.N., Epstein, F.H. 1977. Mechanism of active chloride secretion by shark rectal gland: Role of Na−K-ATPase. *Am. J. Physiol.* **233**:F298–F306

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