TABLE 2 ALANINE PRODUCTION BY PECTORAL FIN MUSCLES INCUBATED IN VITRO

Days Starvation	Number of Experiments	Alanine Content (μmoles/g)				
		In muscle after incubation	In medium after incubation	Total after incubation	In muscle*before incubation	Alanine produced
1	6	1.51±.29	2.71±.36	4.22±.53	1.93±.36	2.29±.31
12-17	6	0.99±.12	3.59±.55	4.58±.66	1.23±.09	3.35±.60
20-22	7	1.05±.07	3.67±.24**	4.72±.23	1.25±.11	3.47±.24**

Incubations were carried out for 90 minutes at 15°C in dogfish Ringer's solution supplemented with glucose (3mM) and isoleucine (3mM). Results are means ± S.E.

*obtained from an adjacent muscle, freeze-clamped before incubation **p<.05 vs. one-day starvation

incubation medium reduced alanine production by muscles from 14-day starved fish to 10.7 ± 4.2% (means ± S n = 4) of the amount produced in its presence. Omission of both isoleucine and glucose lead to the production of only 4.5 ± 4.7% (n = 4) of the alanine produced in their presence. The omission of glucose alc caused no significant change in the rate of alanine production. When leucine (3 mM) was substituted for isoleucine, no significant change in alanine production was observed. Substantially the same results were obtained using fish one day after capture (that is, 'fed' fish). It would appear that for alanine production, an exogenous amino acid is required but that glucose per se is not.

To determine whether the enzyme phosphoenolpyruvate carboxykinase (PEPCK) is involved in alanine synth sis in skeletal muscle, 3-mercaptopicolinic acid, an inhibitor of the enzyme, was tested. With muscles fr 17-20 day starved fish, the inhibitor (1 mM) did not reduce the rate of alanine synthesis significantly. This was so whether isoleucine or leucine was the added amino acid and whether or not glucose was present.

In summary, the above experiments demonstrate that skeletal muscle of the spiny dogfish can synthesize alanine from other amino acids. During starvation the alanine is released into the circulation where it can be transported to the liver and utilized for biosynthesis of urea and glucose. This process helps the fish to maintain blood urea and glucose levels during starvation. Nevertheless, the supply of this amino acid to the liver is insufficient to maintain urea biosynthesis at a normal level during starvation.

This research was supported by NSF Grant PCM 75-14322 and the Holden Fund (Rhode Island Hospital).

TRANSPORT OF SODIUM INTO BRUSH BORDER MEMBRANE VESICLES ISOLATED FROM FLOUNDER INTESTINE AND FLOUNDER KIDNEY TUBULES

Heini Murer, Jill Eveloff, Rolf Kinne, William B. Kinter and Michael Field, Max-Planck-Institut fur Biophysik, Frankfurt, Germany, Mount Desert Island Biological Laboratory, Salsbury Cove, Maine and University of Chicago, Chicago, Illinois

In recent studies on the intact epithelium of the intestine from the winter flounder, *Pseudopleuronect americanus*, it was demonstrated that Cl^- absorption was tightly coupled to the transport of Na^+ (Field and Smith, Bull. MDIBL 15, 1975; Field, Bull. MDIBL 16, 1976). Experiments by Frizzell, Smith and Field (to Bull.) further demonstrated that sodium-coupled chloride entry at the luminal border of the flounder intestine was inhibited by a cAMP-mediated process and also by furosemide. These findings suggest that the Cl^--absorptive mechanism in the teleost intestine may be similar to that in rabbit ileum and gallbladder (Schultz and Frizzell, Biochemistry of Membrane Transport, Springer-Verlag, Neidelberg/New York, 1977).

contrast, in studies with isolated membrane vesicles from rat small intestine and rat proximal tubule we found evidence for the presence of a Na^+/H^+-exchange system (Murer et al. Biochem. J. 154:597, 1976), while no clear evidence for a chloride-coupled sodium entry mechanism could be found in rat small intestine (Murer and Hopfer, unpublished results).

Based on the firm evidence, from in vitro studies with intact flounder intestine epithelium, for the presence of a coupled transport mechanism for sodium and chloride in the luminal membrane, we undertook to study sodium transport in isolated brush border membrane vesicles from flounder intestine. Experiments were carried out to test for the presence of a coupled transport system for sodium and chloride as well as for the presence of a Na^+/H^+ exchange system. Experiments were performed in parallel with membrane vesicles obtained from flounder kidney tubules. The experiments provide evidence for at least two different transport systems for sodium in the flounder intestinal brush border membranes; a coupled Na^+/Cl^- transport system and a Na^+/H^+ exchange system, while studies with renal brush border membrane vesicles showed only the presence of the Na^+/H^+ exchange system.

Brush border membrane vesicles from flounder intestine and flounder kidney were isolated by a calcium precipitation method according to a modification of the procedure described by Schmitz et al. (Biochim. Biophys. Acta 323:98, 1973). Flounder (ca. 300-500 g) were taken from seawater and killed by decapitation. Intestine from about 2.5 cm below the stomach to about 5 cm above the anus was removed, rinsed with a buffer containing 300 mM mannitol and 12 mM Tris/HCl (pH 7.1) mannitol buffer. The mucosa was scraped off from 3-4 everted intestines ($\sim$ 1 g scraped mucosa). Kidneys of 3-4 flounders were excised, chilled in ice-cold mannitol buffer and cut into small pieces with scissors. The tubules were released from the haematopoietic tissue by suction of the tissue suspension through a syringe and then separated by low speed centrifugation (Eveloff et al. Bull. MDIBL, 16, 1976). Intestinal mucosa or renal tubules (1 g tissue/6 ml buffer), respectively, were diluted 6 times with ice-cold distilled water and homogenized for 2 min at full speed in a Waring Blender. $CaCl_2$ was added to a final concentration of 30 mM and after 20 min the suspensions were centrifuged for 15 min at 3,000 g. The pellet was discarded and the remaining supernatant centrifuged at 27,000 g for 30 min. The clear supernatant was discarded and the pellets were suspended in 40 ml of a buffer containing 100 mM mannitol, 1 mM Ca^{++}-gluconate and 20 mM Tris-HEPES (pH 8.2). After homogenizing in a glass/Teflon potter ($\sim$ 1,000 rev./min, 10 strokes) the suspension was passed through cheesecloth and centrifuged at 27,000 g for 30 min. The final pellet was resuspended in about 300 µl of the pH 8.2 buffer, yielding a protein concentration of 5-10 mg/ml. Alkaline phosphatase (marker enzyme for the brush borders) and Na^+-K^+-ATPase (marker enzyme for basal-lateral plasma membranes) were measured as described by Heidrich et al. (J. Cell Biol. 54:232-245, 1972). Transport of labeled substrates was studied by using a Millipore filtration technique (Hopfer et al. J. Biol. Chem. 248:25-32, 1973).

In intestinal and renal membrane preparations alkaline phosphatase activity was enriched by a factor of 6-7, whereas no measurable Na^+-K^+-ATPase could be detected. Sodium-dependent uptake of D-glucose and L-alanine (a specific property of the luminal membrane) could be demonstrated in intestinal and in renal membrane preparations (H. Murer et al., manuscript in preparation). The intravesicular space, which was calculated from the equilibrium uptake of glucose, alanine, mannitol and sodi um varied from 2 to 4 µl/mg protein, but within the same experiment was identical when calculated from the uptake of different solutes. This finding strongly indicates that the substrates are transported into an intravesicular space rather than bound to the membrane.

In Figure 1 the time curves for sodium uptake by brush border membrane vesicles isolated from flounder intestine and kidney are shwon. In the small intestinal membrane preparation (Figure 1A) influx of sodium was faster in the presence of chloride than in the presence of gluconate. Furthermore, the stimulation of sodium flux by chloride was prevented if the membranes were pretreated for 5 min with 0.5×10^{-3} M furosemide and uptake was studied in the presence of 0.5×10^{-3} M furosemide. In the absence of chloride, furosemide

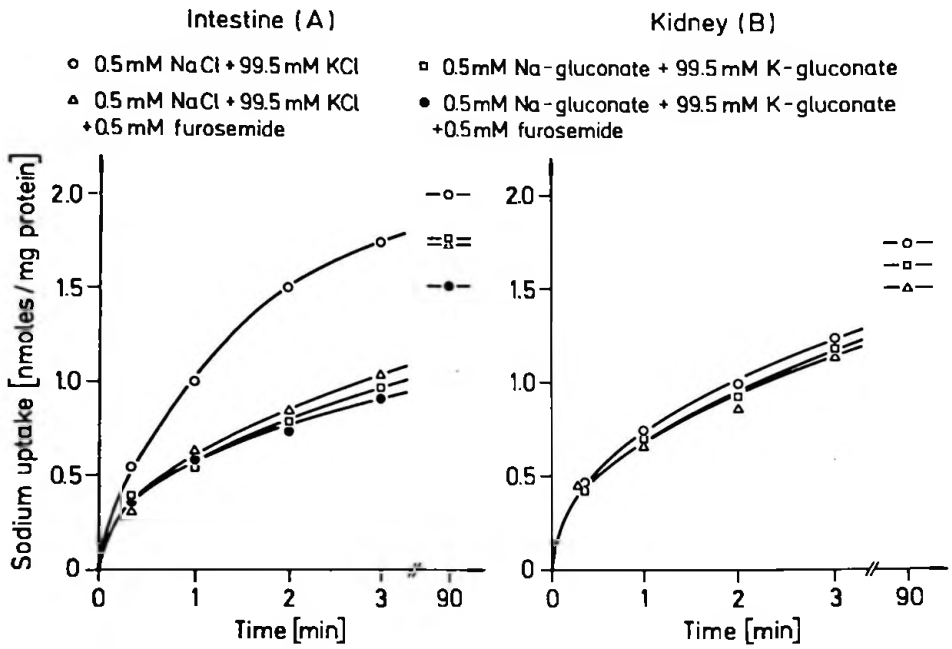

Figure 1. Effect of chloride and effect of furosemide on the uptake of sodium by isolated brush border vesicles. Preincubation and incubation media contained 100 mM mannitol, 2 mM Ca^{++}-gluconate, 20 mM HEPES-Tris (pH 8.2). Incubation media also contained the additions indicated in the figure. To test the effect of furosemide the membranes were preincubated with 0.5 X 10^{-3} M furosemide. The same concentration was also present in the incubated medium. The solution used for the dilution of the samples prior to filtration contained 150 mM mannitol, 20 mM HEPES-Tris (pH 8.2), 100 mM K^{+}-gluconate and 2 mM Ca^{++}-gluconate. The same solution was also used to wash the filters.

did not inhibit sodium uptake. The influx of alanine was not influenced by furosemide (data not shown). In the kidney membrane preparation (Figure 1B), neither the stimulatory effect of Cl^{-} nor the inhibitory effect of furosemide could be detected. Evidence for an uptake of sodium via facilitated diffusion system(s) in the presence and in the absence of chloride was obtained by the tracer replacement experiments shown in Table 1. High sodium concentrations inhibited the uptake of radioisotope in the presence as well as in the absence of chloride. Also tracer coupling experiments (data not shown) provided evidence for a carrier-mediated transport of sodium across the luminal membrane from the intestine as well as from the kidney. Preloading the membrane vesicles with unlabeled sodium stimulated isotope uptake.

Since the anions, chloride and gluconate, were present under gradient conditions in the experiments presented above (Figures 1A and 1B), the stimulation of sodium flux by chloride could also be explained by electrical coupling, since the vesicle membrane diffusion potential would be more negative in the presence of a potassium chloride gradient than in the presence of a potassium gluconate gradient (gluconate is a rather impermeable anion). As shown also in Table 1 a significant stimulation of sodium flux by a chloride gradient was also observed in intestinal membrane preparations when the potassium conductance of the membrane was increased by the addition of the potassium selective ionophore, valinomycin. In this experimental situation the chloride diffusion potential would be at least partly compensated by potassium movements via the valinomycin system. Table 1 therefore provides strong evidence for the presence of a coupled system for sodium and chloride in the intestinal brush border membrane. The small inhibition of sodium

TABLE 1

Effect of membrane potential on sodium uptake by isolated brush border membrane vesicles: Tracer replacement

Additions	^{22}Na Uptake/mg protein/1 min (cpm)	
	Intestine	Kidney
1 mM NaCl + 100 mM KCl	17990	15910
1 mM NaCl + 100 mM KCl + valinomycin	14790	14890
1 mM Na^+-gluconate + 100 mM K^+-gluconate	12890	15630
1 mM NaCl + 100 mM NaCl	11100	10510
1 mM Na^+-gluconate + 100 mM Na^+-gluconate	9520	10730

Uptake of sodium was studied in a medium containing 100 mM mannitol, 2 mM Ca-gluconate, 20 mM HEPES-Tris (pH 8.2) and the additions as indicated in the table. Valinomycin was added as ethanolic solution (final concentration was ~ 5 µg/mg protein). All incubation media contained 1% ethanol.

uptake observed after addition of valinomycin in the presence of a KCl gradient is most probably due to a more rapid dissipation of the chloride gradient (via sodium-independent rheogenic pathways) in the presence of valinomycin. Thus the driving force for the sodium-chloride co-transport system is reduced. Indeed preliminary experiments with $^{82}Br^-$ on the anion conductivity of the luminal membrane showed a 30-40% stimulation of $^{82}Br^-$ flux in the presence of a potassium gluconate gradient (medium > vesicle) by the addition of valinomycin. In renal membranes, valinomycin has no effect on sodium movement, as expected from the anion-independent sodium movement described above.

The results presented in Figure 2 provide evidence for an additional pathway for sodium in the renal and intestinal luminal membranes. In these experiments sodium influx was studied in the presence of a pH-gradient across the membrane. A proton gradient directed in the same direction as the net tracer sodium flux inhibited sodium uptake markedly. As can be seen from the (sodium-independent) alanine influx no non-specific leakage of the membrane is produced by these maneuvers. The inhibition of sodium influx by a proton gradient directed from medium to the vesicle inside is best explained by the operation of a sodium-proton exchange system in the membrane as was described earlier for rat renal and intestinal brush border membranes. Indirect coupling of proton and sodium flux via a proton diffusion potential is unlikely since so far there is no evidence for rheogenic sodium movement across the renal brush border membrane and, if present at all in the intestinal brush borders, this pathway is quite small. In contrast to the chloride-coupled transport system for sodium the Na^+/H^+ exchange system seems to be present in the intestinal as well as in the renal brush border membranes.

Supported by NIH Grants AM 15973, AM 21345 and Max-Planck Institut für Biophysik, Frankfurt, West Germany.

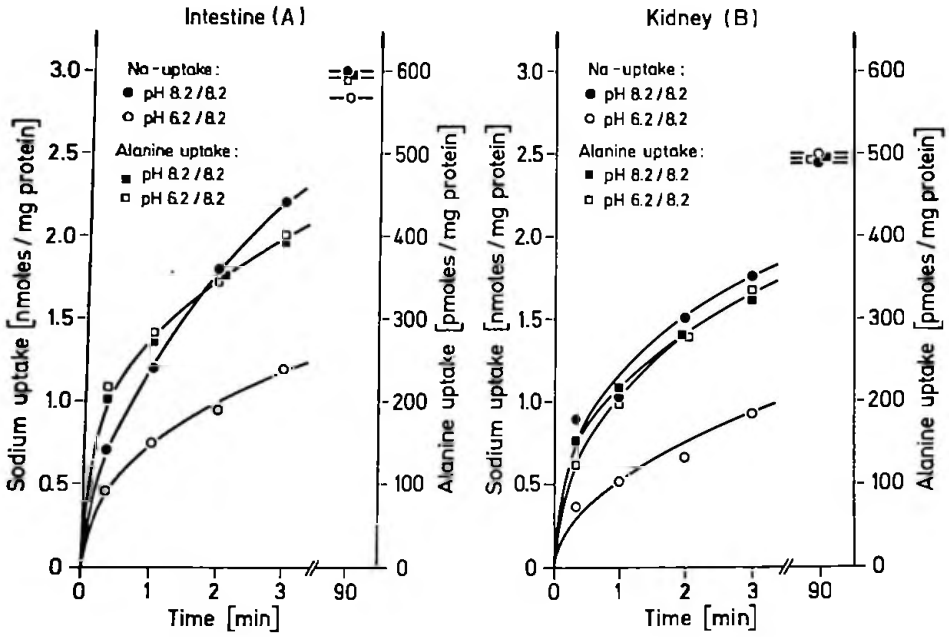

Figure 2. Effect of a pH-gradient on sodium uptake by isolated brush border vesicles. Preincubation median and dilution and wash solution were those indicated in the legend to Figure 1. The incubation media contained 100 mM mannitol, 0.5 mM $^{22}NaCl$, 99.5 mM choline chloride, 2 mM Ca^{++}-gluconate and either 20 mM HEPES-Tris (pH 8.2) or 20 mM MES-Tris (pH 6.2).

INHIBITION OF CALCIUM-DEPENDENT ATPase FROM MOLLUSC MANTLE TISSUE

Gaylen J. Neufeld, John B. Pritchard, and Calvin U. Cotton, Laboratory of Pharmacology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina

It has been well documented that in many species of birds, exposure to organochlorine pesticides leads to the formation of thin eggshells which are easily broken, thus leading to reproductive failure (Nature 224:44-46, 1969; Nature 224:47-48, 1969; Comp. Gen. Pharmacol. 4:305-313, 1973). An enzymatic basis for the thinning effect has been proposed by Miller et al. (Nature 259:122-124, 1976). They have shown that eggshell-thinning in ducks was accompanied by decreased activity of the calcium-transporting enzyme, Ca-ATPase, in the shell gland.

The process of shell formation by the mantle tissue in molluscs can be considered analogous to that occurring in the shell gland of birds. The purpose of this study was to determine if DDT, in vitro also inhibited the Ca-ATPase from mantle tissue of several species of molluscs.

Clams, *Mya arenaria*, and mussels, *Mytilus edulis*, were collected on Laboratory Beach, Salsbury Cove, Maine. Sea scallops, *Placopecten magellanicus*, were kindly supplied by Dan Schick, Department of Marine Resources, Boothbay Harbor, Maine. Ca-ATPase activity was determined by a modification of the method of Miller et al. (Nature 259:122-124, 1976). The final concentrations in the assay medium (1.5 ml) were 10 mM $CaCl_2$, 2 mM disodium ATP, and 92 mM Tris (pH 7.4). DDT was added to the assay medium in dimethyl sulfoxide. The final solvent concentration was 1.6%.

The results from these experiments are shown in Figure 1. Baseline control values for clam and scallop Ca-ATPase were 3.45 and 2.65 μmoles P_i/mg protein/hr, respectively. In contrast, the mussel has a much