J. exp. Biol. (1981), 90, 123–142
With 9 figures
Printed in Great Britain

Na AND Cl TRANSPORT ACROSS THE ISOLATED ANTERIOR INTESTINE OF THE PLAICE *PLEURONECTES PLATESSA*

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(*Received* 25 *February* 1980)

SUMMARY

1. The tissue was found to have a serosa negative potential, and short-circuit currents equivalent to the net Cl transport. 2. A significant part of the Cl uptake was Na dependent and a similar fraction of the Na uptake was Cl dependent. 3. Short-circuit current and uptake of both ions were inhibited by loop diuretics and analogues. 4. I_{sc} and P.D. were abolished by ouabain. 5. The observations are consistent with the idea of a coupled NaCl entry into the cell, using the energy inherent in the Na gradient; Na being pumped out of the cells by the Na pump and followed electrically by Cl^-. Net chloride transport and the serosa negative potential would be a consequence of the permselective properties of the junctions allowing Na but not Cl to recycle back to the mucosal solution.

INTRODUCTION

The mechanism of chloride transport in epithelia has not yet been fully elucidated although it is a dominant process in many tissues, including the thick ascending limb of the loop of Henle (Burg & Green, 1973; Kokko, 1974), cornea (Zadunaisky, 1966), gastric mucosa (Hogben, 1951), marine teleost gill (Maetz & Bornancin, 1975) and anterior intestine (Huang & Chen, 1971). In other tissues, active Cl transport has been described: for influx in squid axon (Russell, 1979), and for efflux in barnacle giant muscle fibre (Russell & Brodwick, 1979).

An electrogenic Na-independent Cl pump has been postulated for the intestines of certain teleosts, such as eel and flounder, which sustain a serosa negative potential difference *in vitro* (Huang & Chen, 1971; Ando, 1975; Ando, Utida & Nagahama, 1975; Hirano *et al.* 1976; Smith, Ellory & Lahlou, 1975) and transport appreciably more Cl than Na under short-circuit conditions (Ando *et al.* 1975). However, the evidence is not entirely convincing. For example, in the study of Huang & Chen (1971), the replacement solutions used to evaluate the role of Na contained 25 mM-Na (as HCO_3) and were therefore not Na-free. In addition, an electrically neutral, coupled NaCl absorption has been proposed for *Cottus scorpius* (House & Green, 1965) and the flounder (Field *et al.* 1978). This uptake would resemble that of rabbit gallbladder (Frizzell, Dugas & Schultz, 1975) and small intestine (Nellans, Frizzell & Schultz, 1973). Field *et al.* (1978) propose that much of the Na transported into the

lateral intercellular spaces in the flounder intestine can recycle to the mucosal solution via Na-selective 'tight junctions', thereby reducing transepithelial Na transport to a fraction of that for Cl, and resulting in a serosa negative potential.

In the present study we have investigated Cl transport in anterior intestine of plaice *Pleuronectes platessa*, by measuring bidirectional Na and Cl transepithelial fluxes, electrical parameters and unidirectional Na and Cl uptake from the mucosal solution into the epithelium.

METHODS

Animals

Plaice *Pleuronectes platessa* weighing from 200 g to 3000 g were caught in the English Channel and North Sea, by Marine Biological Laboratories, Plymouth, and Fisheries Research Laboratories, Lowestoft. They were kept unfed in sea water tanks at 8 °C for more than one week before use.

Experimental procedure

The fishes were killed by decapitation, and after opening the abdomen, the entire intestine was removed. The intestine was opened longitudinally, mounted in Ussing or Schultz type chambers and washed with saline. The saline solution was continuously gassed with 95% O_2–5% CO_2 gas mixture and contained 130 mM-NaCl, 1·1 mM-$MgSO_4$, 10 mM-KCH_3COO, 2·5 mM-$CaCl_2$, 25 mM-choline HCO_3, 10 mM-glucose, and 2 mM-alanine (pH 7·4 adjusted with Tris-base (Tris (Hydroxymethyl) aminomethane) when necessary. All experiments were carried out at room temperature about 20 °C.

Electrical measurements

Four pieces of tissue from each intestine were mounted for simultaneous studies in Ussing type chambers where the area of exposed tissue was 0·7 cm^2 and the volume of each half chamber 1 ml. P.D. (transepithelial potential difference) measurements were made with 3% agar-saline bridges, inserted 1 mm from each surface of the membrane, and connected to a pair of calomel electrodes. Ag/AgCl electrodes were used for passing current through the tissue using an automatic voltage clamp, which also compensated for the solution resistance between the potential difference bridges.

The P.D. across the membrane was expressed in terms of the potential of the serosal side with respect to the mucosal side. The direction of the short-circuit current (I_{sc}) from mucosa to serosa was taken as positive. Electrical conductance of the tissue (G) was estimated from the P.D. and I_{sc} since the tissue behaved as a linear d.c. resistor between −60 mV and +60 mV.

The current in some preparations continued to decline after the initial 30 min equilibrium period. If the current dropped more than 20% between 30 and 60 min following mounting the tissue, it was discarded. Although there was a consistent fall in the P.D. and I_{sc} over the first 30 min both parameters were stable for at least 3h subsequently, the mean values being $-5{\cdot}2 \pm 0{\cdot}2$ mV and -90 ± 4 μA cm ($n = 48$) respectively.

Ion flux measurements

Bidirectional Na fluxes across the short-circuited intestine were measured with ^{24}Na (mucosal side 2 μCi/ml) and ^{22}Na (serosal side 0·2 μCi/ml). Bidirectional Cl fluxes were determined using ^{36}Cl (serosal side 0·2 μCi/ml) and ^{77}Br (mucosal side 0·5 μCi/ml). Preliminary experiments established the identity of ^{36}Cl and ^{77}Br as tracers for following Cl movements, and the stability of the preparation. The ratio $^{77}Br/^{36}Cl$ was influx 1·0 ($n=54$), backflux 1·1 ($n=54$).

Initially the preparation was mounted and equilibrated in non-labelled solutions, while open-circuit potential and short-circuit current were monitored. After 15 min the non-labelled saline was replaced by isotopically labelled solution. Thereafter these radioactive solutions were replaced every 15 min by fresh radioactive ones, each experiment representing 12 to 16 periods. These experiments were continuously under short-circuit conditions except for a few seconds every 7 min during which spontaneous open-circuit potential was measured. One hour incubation with isotopes was necessary to establish isotopic equilibrium within the tissue. Washout experiments established that isotopic wash had a similar time course. Since there was a lag in the isotopic fluxes due to the tissue isotopic equilibration, fluxes were related to the current measured 1 hour earlier.

^{24}Na was counted as Cerenkov radiation in a β scintillation counter, ^{77}Br by γ scintillation counting and after the decay of either ^{24}Na or ^{77}Br, 3 ml of scintillant fluid (Pico-Fluor™ 30) was added to the vials and ^{22}Na or ^{36}Cl determined by β scintillation in a Packard Tricarb scintillation spectrometer.

All isotopes came from Radiochemical Centre, Amersham, England, except for the ^{77}Br which came from Medical Research Council, Cyclotron Unit, Hammersmith, England.

Inhibitor experiments

Pharmacological agents which were not water soluble (e.g. amiloride, triamterene and phloretin) were dissolved in dimethylsulphoxide (DMSO) and then diluted with saline (DMSO final concentration 1 %).

Drug suppliers

Acetazolamine and Ouabain: Sigma Chemical Company. Amiloride: gift from A. Cuthbert. Bumetanide and 3-*n*-Butylamino-4-chloro-5-sulfamoyl-benzoic acid HH-594: Leo Pharmaceutical Products. Phloretin: K. & K. Laboratories Inc. Furosemide Piretanide and Hoe 740B: Hoechst Pharmaceuticals. 4-Acetamido-4-isothiocyanato-stilbene-2, 2-disulfonic acid (disodium salt) (SITS): BDH Chemicals Ltd. (Thioxo-5-dithiole-1,2,yl-3) 5 thiophene-2 sulphonate triethyl-ammonium TST: gift from J. Maetz. Triamterene: Smith, Kline & French Laboratories Ltd. (6,7-Dichloro-2-methyl-1-oxo-2-Phenyl-5-Imdanyloxyl) Acetic Acid (MK-196) and Chlorothiazide: Merck, Sharp & Dohme Research Laboratories.

pH experiments

Effects of varying pH on I_{80} were tested using solutions of pH 6, 6·8, 7·4, 8·0, 8·6 and 9·0. The experiments were always started at pH 7·4 (control) and substitutions

Table 1. *Effect of HCO_3^- on Na and Cl uptake*

	Uptakes nmol × cm^{-2} × min^{-1}	
	Na	Cl
Na HCO_3 25 mM	395 ± 31 (15)	220 ± 16 (15)
Na isethionate 25 mM	387 ± 34 (15)	198 ± 14 (15)

Each number is the mean ± S.E.M. Number of observations in parentheses. Control solution had NaCl 85 mM and Na HCO_3 25 mM and was gassed with 95 % O + 5 % CO_2 gas mixture. Test solution was not gassed and HCO_3^- was substituted by isethionate 25 mM.

made on both sides of the Ussing chambers to the same pH, each substitution lasting for 20-30 min. The preparation was continuously gassed with 100% O_2 and the ringer used had Hepes (*N*-2-Hydroxyethyl piperazine-*N'* -2-ethano-sulphonic acid) instead of HCO_3 for pH between 6·8 and 8·6, MES (2 [*N*-Morpholino] ethane sulphonic acid for pH 6·0 and BICINE (*N,N*-bis 2[-Hydroxyethyl] glycine) for pH 9·0.

Uptake experiments

Unidirectional Na and Cl influxes were determined using the method first described by Schultz et al. (1967) modified by Ellory & Smith (1970). Isotopic test media contained either ^{24}Na (30 μCi/ml) or ^{36}Cl (10 μCi/ml) or both and ^{3}H-inulin (40 μCi/ml) as a space marker. Some experiments were performed comparing ^{3}H-inulin with either $^{35}SO_4^{2-}$ (10 μCi/ml) or ^{51}Cr-EDTA (10 μCi/ml) as space markers. The areas of exposed tissue in these experiments were either 0·06 cm^2 or 0·13 cm^2. All experiments were performed at room temperature. Tissues were preincubated for 10-30 min in saline and pre-washed with media whose composition was identical to that employed for the subsequent influx determination. The presence of HCO_3 in the test media would necessitate gassing these solutions. This would be made difficult by the small volume of test solutions used in these experiments, so preliminary experiments were performed to test the effect of the removal of HCO_3 from the incubating media on Na and Cl uptakes. The results presented in Table 1 show that the absence of HCO_3 in the test medium does not affect either Na or Cl uptake. Therefore, for practical reasons, all the uptake experiments were performed in the absence of HCO_3 in the incubating media. The basic composition of the influx test media was: 140 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 10 mM-Hepes, and 0·1 mM unlabelled inulin, modified as described in legends to tables. Exposure of the tissue to the isotopic media was for 1 min in all experiments, except when determining time courses.

Statistics

All results are expressed as the mean ± S.E.M.

Statistical comparisons were made using the Student t test; a value of <0.05 was considered significant.

Table 2. *Relationship between electrolyte fluxes and electrical parameters on plaice intestine, measured in normal saline media*

Fluxes	Chloride	Sodium
	(μequiv $\times$ h^{-1} $\times$ cm^{-2})	
J_{m-s}	8·39 ± 0·42 (31)	6·34 ± 0·39 (20)
J_{s-m}	2·99 ± 0·16 (31)	4·94 ± 0·24 (20)
J_{net}	5·40 ± 0·39 (31)	1·41 ± 0·28 (20)
Cl net flux/Na net flux		3·83
Short circuit current		
(I_{sc}) (μequiv X^- $\times$ h^{-1} $\times$ cm^{-2})		3·37 ± 0·16 (48)
(I_{sc}) (μA $\times$ cm^{-2})		−90·2 ± 4·3 (48)
Transepithelial potential		
(P.D.) (mV)		−5·20 ± 0·21 (48)
Total conductance		
(G) (mS $\times$ cm^2)		17·35 ± 0·43 (48)

Average values ± S.E.M. are given for the number of experiments shown in parentheses.

RESULTS

Relationship of isotopic fluxes to electrical measurements

Table 2 presents data relating Na and Cl fluxes to the measured short-circuit current and P.D. in normal saline media. Both sodium influx (J_{ms}^{Na}) and backflux (J_{sm}^{Na}) were large, with net sodium transport (J_{net}^{Na}) being small and subject to greater experimental error. In contrast chloride net transport (J_{net}^{Cl}) was large, with chloride influx (J_{ms}^{Cl}) being three-fold greater than Cl backflux (J_{sm}^{Cl}).

Effect of piretanide and ouabain on electrical parameters

Piretanide rapidly and irreversibly inhibited transepithelial P.D. and I_{sc}, but did not affect conductance, when added to the mucosal solution (Fig. 1, Table 3). Although doses as low as 0·01 mM were maximally effective, they were slow in reaching full inhibition and we routinely used 0·1 mM piretanide to allow complete inhibition within 15 min.

A significant fraction of I_{sc} was piretanide-insensitive (46±6%) and concentrations up to 10^{-3} M were not found to be more effective.

Ouabain (0·2 mM) added to the serosal side did not significantly affect the conductance (Table 4), whilst the I_{sc} and P.D. dropped to virtually zero (Fig.2).

Comparing Figs. 1 and 2 it can be seen that the effect of piretanide on P.D. and I_{sc} is more or less immediate but ouabain takes about half an hour to start acting (which is probably the diffusion time through the muscle layer since this drug is added on the serosal side). Data from Figs. 1-6 are summarized in Tables 3 and 4 to allow statistical comparisons of the effects of the drugs on both electrical and flux data.

Effect of piretanide and ouabain on transepithelial electrolyte fluxes

Cl fluxes

Piretanide causes an inhibition of Cl net flux (Fig. 3) which can be attributed solely to the inhibition of the Cl influx.

In the presence of ouabain (Fig. 4) there is also an inhibition of the Cl net flux but, in this case, the increase in the Cl backflux seems to be of major importance.

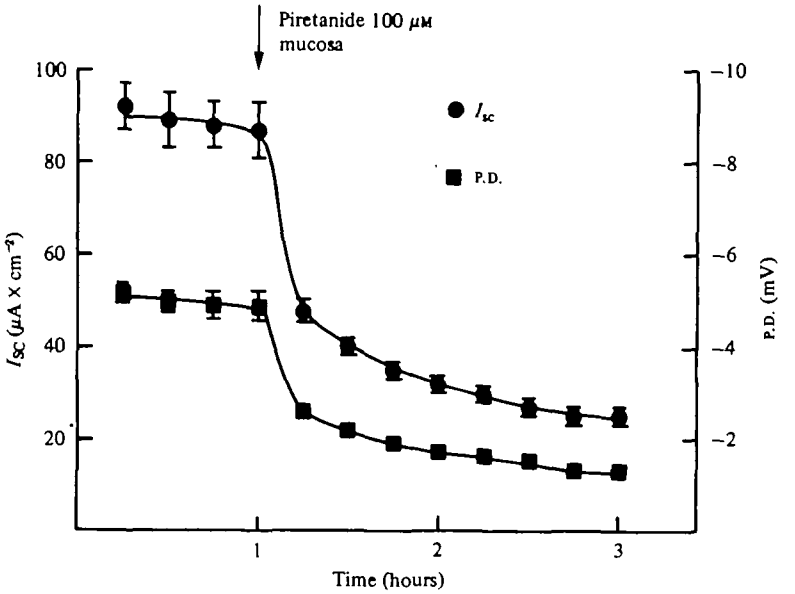

Fig. 1 Effect of piretanide (0·1 mM) on I_{sc} and P.D. Each point is the mean ± S.E.M. of 31 determinations.

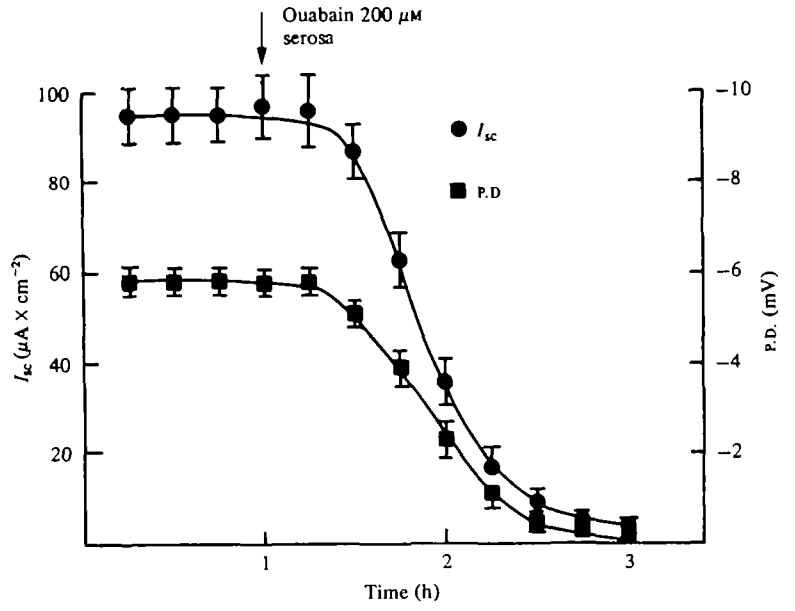

Fig. 2. Effect of ouabain (0·2 mM) on I_{sc} and P.D. Each point is the mean ± S.E.M. of 17 determinations.

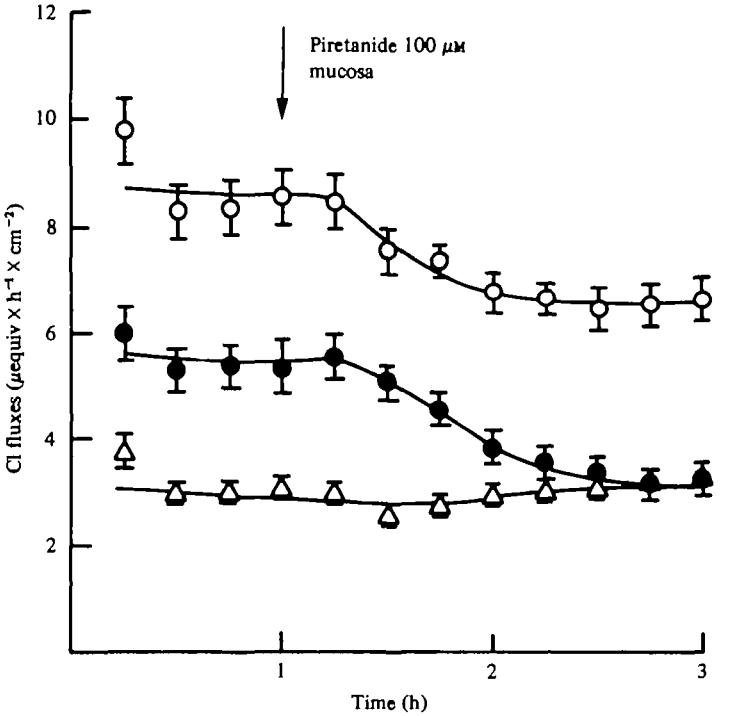

Fig. 3. Effect of piretanide (0·1 mM) on Cl fluxes. ○, Influx; Δ, backflux; ●, net flux. Each point is the mean ± S.E.M. of 22 determinations.

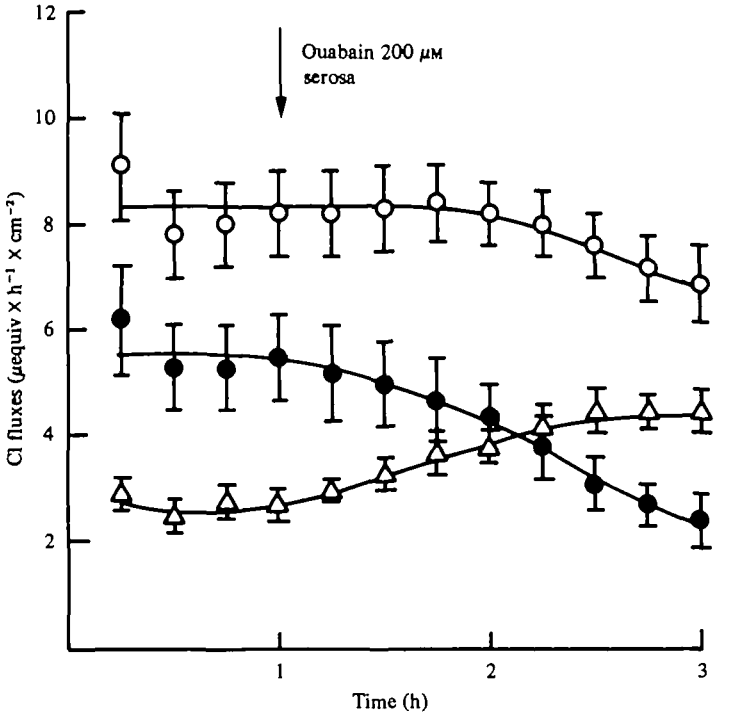

Fig. 4. Effect of ouabain (0·2 mM) on Cl fluxes. ○, Influx; Δ, back flux; ●, net flux. Each point is the mean ± S.E.M. of 9 determinations.

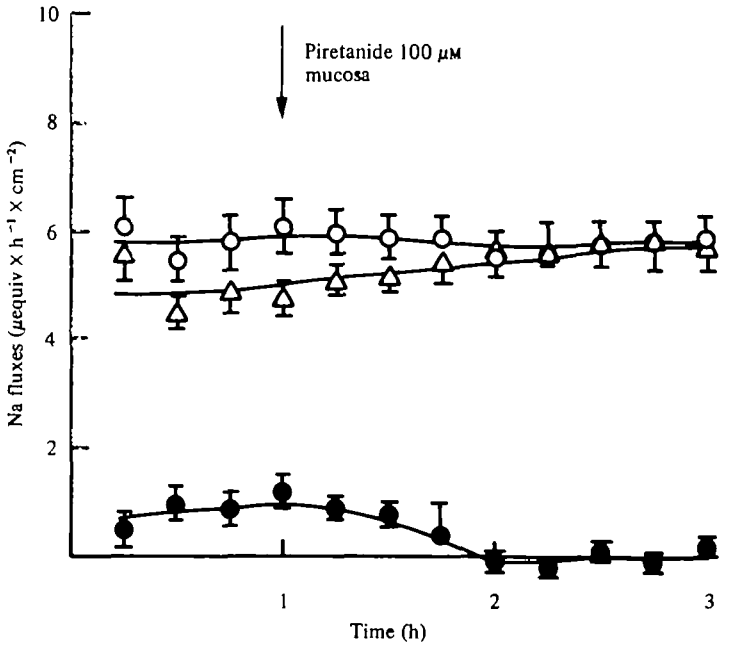

Fig. 5. Effect of piretanide (0·1 mM) on Na fluxes. ○, Influx; Δ, backflux; ●, net flux. Each point is the mean ± S.E.M. of 10 determinations.

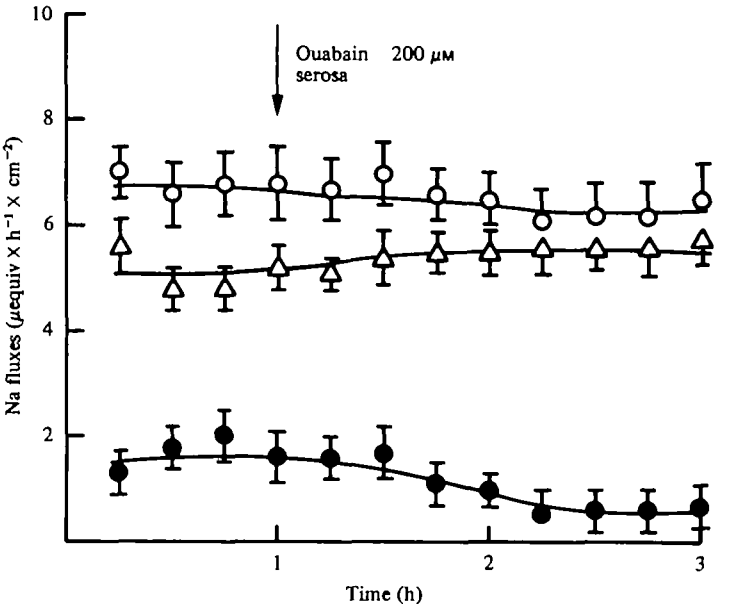

Fig. 6. Effect of ouabain (0·2 mM) on Na fluxes. ○, Influx; Δ, backflux; ●, net flux. Each point is the mean ± S.E.M. of 10 determinations.

Table 3. *Effect of piretanide on the electrical parameters and Na and Cl transport.*

	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	J^{Cl}_{ms}	J^{Cl}_{sm}	J^{Cl}_{net}	I_{sc} ($\mu A \times cm^{-2}$)	I_{sc} ($\mu equiv\ X^- \times h^{-1} \times cm^{-2}$)	G ($mS \times cm^2$)	P.D. (mV)
Control	5·93 ± 0·47	4·88 ± 0·31	1·06 ± 0·26	8·50 ± 0·51	3·09 ± 0·20	5·41 ± 0·44	−87·1 ± 5·5	3·25 ± 0·21	17·8 ± 0·5	−4·9 ± 0·3
Piretanide 10^{-4} M mucosa	5·85 ± 0·43	5·80 ± 0·34	0·04 ± 0·19	6·63 ± 0·39	3·34 ± 0·17	3·28 ± 0·32	−33·5 ± 2·0	1·25 ± 0·08	18·7 ± 0·6	−1·8 ± 0·1
No. of observations	10	10	10	22	22	22	31	31	31	31
Significance	N.S.	N.S.	<0·01	<0·01	N.S.	<0·001	<0·001	<0·001	N.S.	<0·001

All values expressed in $\mu equiv \times h^{-1} \times cm^{-2}$ except for G in $mS \times cm^2$, P.D. in mV and I_{sc} in $\mu A \times cm^{-2}$. Each value represents the mean ± S.E.M.

Table 4. *Effect of Ouabain on electrical parameters and Na and Cl transport*

	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	J^{Cl}_{ms}	J^{Cl}_{sm}	J^{Cl}_{net}	I_{sc} ($\mu A \times cm^{-2}$)	I_{sc} ($\mu equiv\ X^- \times h^{-1} \times cm^{-2}$)	G ($mS \times cm^2$)	P.D. (mV)
Control	6·76 ± 0·62	5·00 ± 0·38	1·76 ± 0·47	8·12 ± 0·77	2·75 ± 0·24	5·37 ± 0·80	−95·9 ± 6·7	3·58 ± 0·25	16·45 ± 0·70	−5·8 ± 0·29
Ouabain 2×10^{-4} M serosa	6·38 ± 0·67	5·73 ± 0·50	0·65 ± 0·41	7·08 ± 0·61	4·52 ± 0·32	2·57 ± 0·41	−49·5 ± 6·8	1·85 ± 0·25	16·74 ± 0·89	−3·1 ± 0·24
No. of observations	10	10	10	9	9	9	17	17	17	17
Significance	N.S.	N.S.	N.S.	N.S.	<0·001	<0·001	<0·001	<0·001	N.S.	<0·001

All values expressed in $\mu equiv \times h^{-1} \times cm^{-2}$ except for G in $mS \times cm^2$, P.D. in mV and I_{sc} in $\mu A \times cm^{-2}$. Each value represents the mean ± S.E.M.

Table 5. *Effect of various pharmacological agents on the* I_{sc}

Drug Group	'Drug'	Concentration (mM)	Inhibition (%)	Time constant (min)	*n*
K^+—sparing diuretics	Triamterene in 1% DMSO	1·0	0	∞	4
	Amiloride	1·0	0	∞	8
	Chlorothiazide	1·0	0	∞	4
	Acetazolamide	5·0	0	∞	7
Benzothiazides	Acelazolamide	5·0	51 ± 3	10 ± 2	3
	Acelazolamide	5·0	91 ± 20 (Stimulation)	3 ± 1	4
Band 3 Inhibitors	SITS	0·3	0	∞	8
	Phloretin in 1% DMSO	0·5	63 ± 4	14 ± 1	7
Loop diuretics	Furosemide	0·1	45 ± 3	3 ± 0	8
	Bumetanide	0·1	39 ± 4	6 ± 2	4
	Piretanide	0·1	60 ± 5	3 ± 0	7
Loop analogues	MK – 196	1·0	37 ± 5	6 ± 1	4
	HH – 594	0·1	49 ± 2	3 ± 0	4
	TST	1·0	38 ± 6	7 ± 1	5
	Hoe 740B	1·0	0	∞	3
Control	DMSO	1%	0	∞	10

The numbers are mean ± S.E.M. See text for further details.

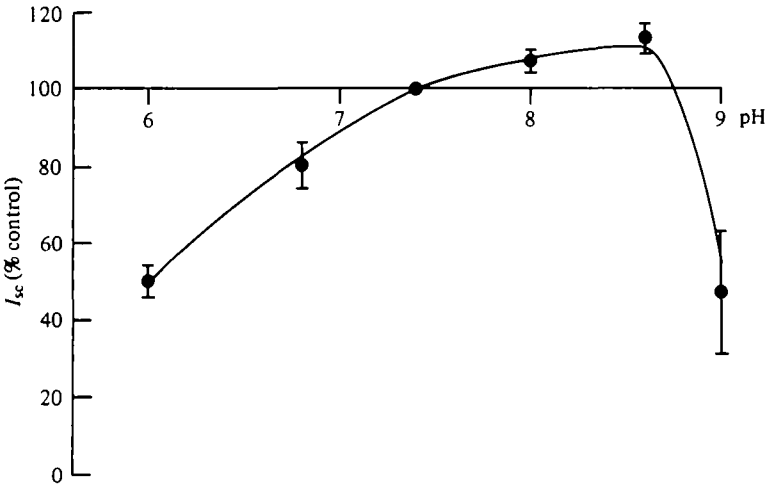

Fig. 7. Effect of pH on I_{sc}. Each point represents the mean ± S.E.M. of the % of the control (pH 7·4) of 7 determinations. See text for further details.

Na fluxes

The influxes of Na and Cl in this tissue are quantitatively similar, but Na backflux is much higher than the Cl backflux giving a small Na transport in contrast with the large net Cl transport. Addition of piretanide has no effect on Na influx, whilst Na backflux tends to increase. Net Na transport is reduced to zero although this is difficult to establish quantitatively since the errors in measuring this parameter (a small difference between two large numbers) are relatively large (Fig. 5). Ouabain abolished net Na transport (Fig. 6) principally by decreasing Na influx although again this is difficult to quantitate.

Considering the data as a whole (Tables 3 and 4), piretanide reduced the short-circuit current and inhibited net Cl transport by an equivalent amount (form Table 3 the short-circuit current was reduced by 54 $\mu A \times cm^{-2}$, equivalent to 2·00 μequiv $Cl^- \times cm^{-2} \times h^{-1}$, and the Cl net flux by 2·13 μequiv $\times cm^{-2} \times h^{-1}$. Na net flux was strongly reduced by piretanide. However it seems that part of the Cl transport may be silent in electrical terms (e.g. in Table 3 $J^{Cl}_{net}-J^{Na}_{net}$ exceeds I_{sc} by 1·10±0·19) and a fraction of the electrogenic Cl transport seems to be piretanide-insensitive.

The effect of several pharmacological agents on I_{sc}

Table 5 shows the percentage inhibition of the current produced by several pharmacological agents, and the time necessary to achieve 50% of this inhibition. None of the effects was reversible by washing and once the steady state was reached, addition of any other of these inhibitors did not have an additive effect.

It is clear that loop diuretics, such as furosemide, bumetanide, piretanide, TST, HH-594 and MK-196 are all effective inhibitors of the Cl-current. Among the effective inhibitors piretanide seems to have the highest affinity. In contrast, band 3 inhibitor SITS and diuretics known to act in other ways (K^+-sparing diuretics and chlorothiazide) were ineffective.

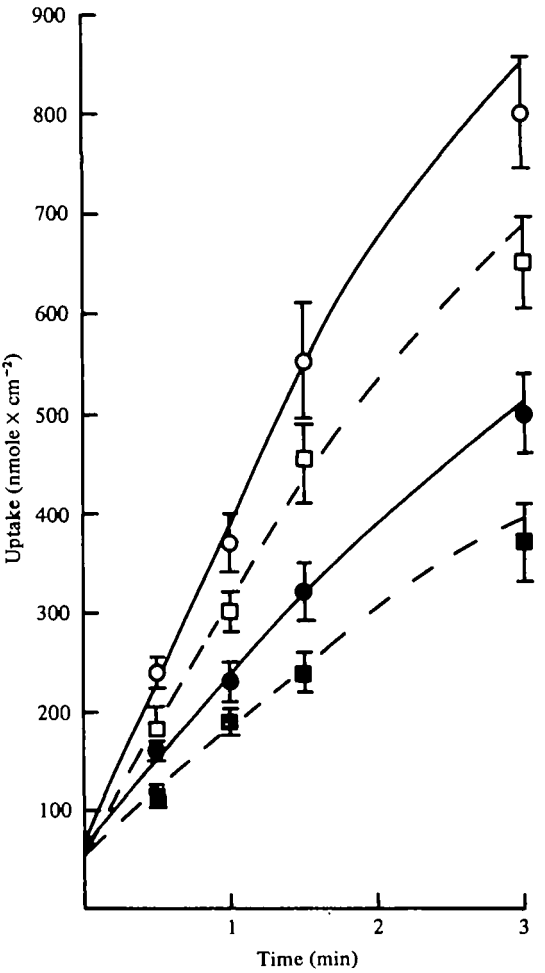

Fig. 8. Time course of the uptake of Na and Cl by plaice intestine. Data presented are mean ± S.E.M. of 16 determinations for the control or 14 determinations in the presence of piretanide (1 mM) on 8 plaice. ○, Na control; □, Na in the presence of piretanide; ●, Cl control; ■, Cl in the presence of piretanide.

Acetazolamide gave very inconsistent results: different individual experiments showing stimulation, inhibition or no effect. Phloretin which has been described as an inhibitor of anion transport (Fortes 1977) and sugar transport in red cells (Le Fevre, 1961) had a strong inhibitory effect on the I_{80} (Table 5).

Thiocyanate (40 mM) had no inhibitory effect on I_{80}. Control experiments showed that reducing Cl concentration in the bathing solution by 40 mM (from 130 to 90 mM) did not affect the I_{80}.

pH experiments

Since it was necessary to exclude HCO_3^- from experiments where the pH was raised, preliminary experiments tested the effect on the I_{80} of removing HCO_3^- at a constant pH (7·4). This gave a 24±1 % (n=7) inhibition of the current.

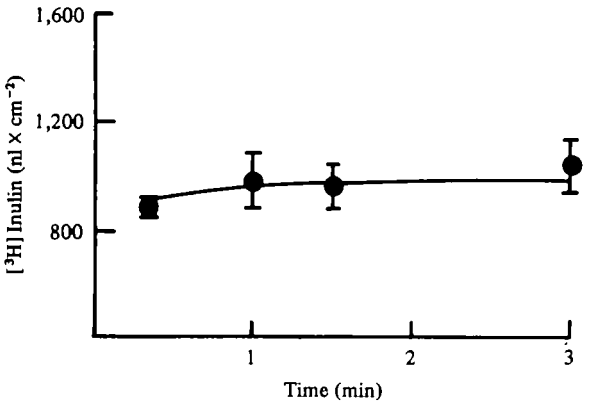

Fig. 9. Space given by [^{3}H]inulin as a function of incubation time. Data are mean ± S.E.M. of 14 observations.

Fig. 7 shows the effect of pH on the I_{sc}, as a percentage of the control value (pH 7·4). When the bathing solutions were at pH below 6·8 or above 8·6 the I_{sc} fell to virtually zero, without any new steady state. Between pH 7 and 8, higher currents were achieved, 7·3–7·6 being the best pH range for this preparation.

Unidirectional Na and Cl influxes

The results of experiments to determine the time course of Na and Cl uptake across the mucosa from saline and in the presence of piretanide 1 mM are presented in Fig. 8. Na uptake was always greater than Cl up to 3 min. The data up to 1·5 min are best fitted by straight lines which do not pass through the origin but give a positive intercept (Fig. 8) of nearly 70 nmol × cm^{-2}.

In order to test whether inulin was under-estimating the excluded space, two types of experiments were performed: (*a*) the use of ^{35}sulphate and ^{51}Cr-EDTA as space markers, comparing the results given by them with the simultaneous space given by ^{3}H-inulin during 1 min incubation, and (*b*) the measurement of the space given by ^{3}H-inulin against time.

The results of the experiments where ^{3}H-inulin, ^{51}Cr-EDTA and ^{35}sulphate were compared showed that ^{51}Cr-EDTA gives a space that is not significantly different from the one given by ^{3}H-inulin: ^{51}Cr-EDTA/^{3}H-inulin = 0·94 ± 0·03 (16). For ^{35}sulphate the difference obtained may represent uptake of this ion: $^{35}SO_4^{2-}$/^{3}H-inulin = 1·38 ± 0·05 (7).

Fig. 9 shows that the space given by ^{3}H-inulin does not significantly change during the three minutes, suggesting that Na and Cl uptake values obtained during the first 1·5 min were not overestimated and that the intercept may represent a space available to Na and Cl but not to inulin.

Although this simple uptake technique has severe technical limitations, including unstirred layer effects, the choice of space markers and the use of open circuit conditions, it is the present intention to look at the *relative* magnitude of Na and Cl fluxes, and in particular the effects of piretanide on uptake rates.

The data presented in Table 6a strongly suggest that approximately 20–30% of the

uptake of Na and Cl is coupled and that the inhibitory effect of piretanide is restricted to this coupled process. These findings cannot be attributed to changes in the electrical potential difference (P.D.) across the brush border, since any effect of piretanide on the P.D. would have opposite effects on the movements of Na and Cl. Nor can the effects be readily attributed to changes in passive Na or Cl influx since piretanide has no effect on the conductance.

The fact that piretanide inhibited a fraction of both Na and Cl by about the same magnitude suggests then that this drug may have a direct effect on the mucosal entry step for Na and Cl.

Table 6. *Effect of various pharmacological agents on Na and Cl influxes across the mucosal membrane*

(*a*)	Uptakes nmol × cm^{-2} × min^{-1}	
	Na	Cl
Control		195 ± 9 (16)
SITS 3 × 10^{-4} M		195 ± 9 (16)
Δ SITS		—
Control	312 ± 25 (16)	199 ± 19 (16)
Phloretin 5 × 10^{-4} M in 1 % DMSO	300 ± 19 (16)	209 ± 20 (16)
Δ Phloretin	12 ± 31 (16)	—
Control	406 ± 22 (42)	250 ± 14 (42)
Piretanide 10^{-3} M	324 ± 16 (42)	194 ± 9 (42)
Δ Piretanide	82 ± 14 (42)	56 ± 10 (42)
Control		186 ± 12 (15)
Bumetanide 10^{-3} M		117 ± 11 (15)
Δ Bumetanide		69 ± 16 (15)

(*b*)	Uptakes nmol × cm^{-2} × min^{-1}	
	Na	Cl
Control	238 ± 21 (16)	183 ± 15 (16)
SCN^- 4 × 10^{-3} M	241 ± 21 (15)	114 ± 17 (16)
Δ SCN^-	—	69 ± 23 (16)
SCN^-	357 ± 20 (32)	89 ± 7 (40)
SCN^- + Piretanide	289 ± 24 (31)	85 ± 16 (38)
Δ Piretanide	68 ± 16	—
Piretanide	243 ± 15 (16)	174 ± 18 (16)
Piretanide + SCN^-	215 ± 23 (14)	89 ± 12 (14)
Δ SCN	28 ± 13	85 ± 11

Each number represents the mean ± S.E.M. Number of observations in parentheses. Inhibitor concentration: piretanide and bumetanide (1 mM), SITS (0·3 mM); Phloretin (0·5 mM); SCN^- (40 mM). For thiocyanate experiments, the basic medium was changed as follows: instead of using NaCl 140 mM, the control solution was prepared with NaCl 100 mM and Na isethionate 40 mM. In the test solution isethionate was substituted by SCN^-.

SITS and phloretin, well known inhibitors of the anion exchange system in red blood cells, had no effect on the Cl uptake by the plaice small intestine (Table 6). Phloretin had no effect on Na uptake either. Bumetanide, a known loop diuretic seems to be as effective as piretanide as an inhibitor of the Cl uptake (Table 6).

Replacement of one third (40 mM) of the Cl by SCN^- inhibited Cl uptake by about 40%, whilst Na uptake was unaffected (Table 6b). The addition of piretanide following SCN^- gave no further inhibition on Cl uptake, whilst thiocyanate gave an additional inhibition following piretanide. These results suggest that SCN^- may be competing with Cl and being transported via the same routes. This effect is clearly different from the specific inhibition achieved with piretanide, since thiocyanate is interacting with more than one entry step and not affecting the Na uptake, suggesting that SCN^- is effectively transported by the coupled piretanide-sensitive route.

DISCUSSION

The anterior intestine of plaice, in vitro, shows a serosa negative potential and a short-circuit current equivalent to the net absorption of chloride (Table 2). Results obtained with ion-sensitive microelectrodes demonstrate that Cl^- is accumulated intracellularly against its equilibrium potential (Zeuthen, Ramos & Ellory, 1978; Ellory, Ramos & Zeuthen, 1979).

Four general classes of Cl^- transporting mechanisms have been proposed for animal tissues.

1. It has been suggested (Kasbekar & Durbin, 1965) that an anion-sensitive Mg-ATPase, and carbonic anydrase are involved in the gastric acid secretion process. Bicarbonate and chloride together would stimulate the ATP-ase leading to phosphorylation by ATP, and to inward bicarbonate and outward chloride transport. Bicarbonate plus a proton would result from the hydration of CO_2 by the carbonic anhydrase. The proton and chloride ion would constitute HCl secretion.

Since then several studies of the anion-sensitive Mg-ATPase in gastric mucosa and other tissues have been made (e.g. stomach, Sachs 1970; kidney proximal tubule, Kinne-Saffran & Kinne, 1974; fish gill, De Renzis & Bornancin, 1977).

The properties of this anion stimulated ATPase and the mitochondrial ATPase strongly resemble each other. Since mitochondria are abundant in these transporting epithelia, contamination of a plasma membrane fraction with mitochondrial fragments cannot easily be prevented. Bonting, van Amelsvoort & de Pont (1978) have claimed that rabbit gastric mucosa, rainbow trout gill, rabbit kidney, and rat pancreas contain only a mitochondrial anion sensitive ATPase, and not a plasma membrane located activity.

2. A Cl^-/anion exchange mechanism has been suggested to operate in the cornea epithelium where Bentley & Yorio (1978) proposed an exchange of Cl for metabolically produced organic anions.

Cl^- exchanging for HCO_3^- has been proposed as an exchange mechanism located on the mucosal border in the gills of fresh water eels (Maetz, 1971). The band 3 anion exchange protein is an obvious analogy for this system and a more general distribution of this transport in epithelia has been proposed (Gunn, 1979). Results consistent with the existence of an anion exchange transport system which depends on metabolic energy and which secretes HCO_3^- in exchange for absorbed Cl^- were presented by Husted, Cohen & Steinmetz (1979) in turtle bladder. Several investigators have suggested Cl^-–HCO^- exchange as the mechanism of HCO_3 secretion in rabbit ileum (Hubel, 1969; Swallow & Code, 1967; Turnberg *et al.* 1970; and Kinney & Code, 1964).

3. Turnberg *et al.* (1970) have suggested the double exchange of Cl-HCO_3 and Na-H as the overall regulatory mechanism for acid-base balance in human ileum.

This third model with Na entering the cell exchanging with protons or K^+ followed by Cl exchanging with an anion has been proposed in rat kidney proximal tubule and small intestine (Pitts, Ayer & Schiess, 1949; Pitts, 1961; Murer, Hopfer & Kinne, 1976) as well as frog skin *in vitro* (Alvarado, Bietz & Mullen, 1975; Garcia-Romeu & Ehrenfeld, 1975; Emilio & Menano, 1975), gills (Motais & Garcia Romeu, 1972), gall bladder (Whitlock & Wheeler, 1969) and urinary bladder of the toad (Frazier & Vanatta, 1971) and turtle (Green, Steinmetz & Frazier, 1968).

4. The fourth type of model proposes a coupled NaCl neutral influx through a membrane carrier, using the downhill movement of sodium along its electrochemical gradient.

This system has been described for rabbit ileum (Nellans *et al.* 1973), gall bladder (Frizzell *et al.* 1975), flounder intestine (Field *et al.* 1978) kidney proximal tubule (Spring & Kimura, 1978), and shark and dogfish rectal gland (Silva *et al.* 1977; Eveloff *et al.* 1978, respectively).

The effects of different inhibitors upon transport in the plaice intestine were studied in an attempt to distinguish between these different systems, since although inhibitor binding may mimic substrate binding and thus give certain common effects, it is likely that specificity of individual transport systems may differ. The models may be appraised in the light of these results, as follows. (1) The fact that SCN^- had no effect on I_{sc} makes it unlikely that an anion sensitive ATPase of the kind described by Kasbekar & Durbin is responsible for the active Cl^- transport described in this preparation. (2) The second proposed model – Cl/anion (probably HCO_3^-) exchange was assessed by the removal of CO_2 and HCO_3^- in the bathing solutions and/or the use of known inhibitors of the Cl^- exchange mechanism in red cells (SITS, phloretin and acetazolamide) or muscle (SITS and SCN^-).

The absence of HCO_3^-, although reducing I_{sc} by about 24%, had no effect on either Na or Cl uptake (Table 1). This inhibitory effect on the I_{sc} might be explained by an inhibitory action on the Cl exit from the cell through the basolateral membrane. If these membranes are impermeable to both Cl and HCO_3^- and the Cl exit from the cell is accomplished by an exchange mechanism with HCO_3^-, the removal of HCO_3 from the bathing solution will reduce the rate of exchange.

SITS is a disulfonic stilbene which Cabantchik & Rothstein (1972) found to block the carrier-mediated exchange of anions across erythrocyte membranes. Ehrenspeck & Brodsky (1976) in turtle bladders showed that the anion-dependent (HCO_3^-, Cl^-) component of the short circuit current was eliminated by SITS. In barnacle muscle fibre, Boron, Russell & Brodwick (1978) presented evidence for a Cl^-–HCO_3^- exchange as a pH regulatory mechanism, which is inhibited by SITS and furosemide. They further suggested that this carrier could mediate a Cl-Cl exchange as well. Russell & Brodwick (1979) and Ashley *et al.* (1978) showed that this Cl-Cl exchange system is partially inhibited by SITS. In short-circuited turtle bladder in the presence of HCO_3^-, unidirectional and net Cl fluxes are not altered by serosal or mucosal SITS addition (Husted *et al.* 1979). This result is in agreement with the present work, since in this preparation SITS had no effect either on I_{sc} or on the Cl uptake.

Phloretin, which interacts with the anion transport protein in red cells, and is also

an inhibitor of the sugar transport system in red cells (Le Fevre, 1961), strongly inhibited the I_{sc} (about 63%) but had no effect on either Na or Cl uptake in plaice anterior intestine.

Acetazolamide affected I_{sc} very inconsistently.

Besides the inhibitory effects on the 'anion stimulated' ATPase, thiocyanate has been found to have inhibitory properties on halide exchange transport in tissues such as thyroid (Wolff, 1964), gastric mucosa (Forte, 1972), cornea (Zadunaisky, Lande & Hafner, 1971), and fish gills (De Renzis, 1975). In the present study, SCN^- appeared to be interacting with more than one entry step and appeared to be effectively transported by the piretanide-sensitive route. This idea is supported by the observations of Cremaschi, Henin & Meyer (1979) who suggested that, in rabbit gall bladder, SCN^- competes with Cl^- for the cotransport with Na^+ with a consequent abolition of Cl^- entry into the cell and the accumulation of this drug inside the cell. As a result of these studies it is concluded that a Cl/anion exchange mechanism is not the main process responsible for the active Cl transport in plaice intestine. (3) The third model–Na^+/H^+ or K^+ exchange followed by Cl^-/anion exchange–is partly ruled out by the reasons that excluded the above discussed model.

Inhibitors of Na permeability via the specific Na channel in high resistance epithelia–triamterene and amiloride– had no effect on this preparation confirming previous results where these drugs had no effect on leaky epithelia (Henriques de Jesus, 1974).

The thiazide diuretic, chlorothiazide, which inhibits the Na^+–H^+ process in proximal tubules and red cell chloride exchange but does not affect carbonic anhydrase (Brooks & Lant, 1978) also has no effect in plaice intestine.

If the limiting step is the amount of H^+ and HCO_3^- that can exchange for Na^+ and Cl^-, then an increase of the intracellular pH could stimulate I_{sc}, since more HCO_3^- would be available leading to a higher rate of Cl^-/HCO_3^- exchange. This was not seen in the present study (Fig. 7). However, in the eel intestine, P.D. and I_{sc} augment when the pH of the mucosal solution is raised to pH 9 or 10 (Hirano *et al.* 1976; Oide, 1973). (4) Although the present results do not allow an unequivocal description of the Cl transport system in plaice intestine the data strongly suggests the fourth proposed model: a coupled Na Cl neutral influx across the mucosal membrane coupled to the downhill movement of Na along its electrochemical gradient.

The results have shown that the loop diuretics: furosemide, piretanide, bumetanide, MK-196 and HH-594, all of them inhibiting active Cl transport in the kidney and Cl transport in red blood cells (Brooks & Lant, 1978) inhibited the I_{sc} in this preparation in a very similar way (Table 5). Piretanide inhibits Na and Cl uptake by approximately the same amount (Table 6*a*). Piretanide has no effect on the conductance of this tissue. Its inhibitory action cannot be explained by changes in the P.D. across the brush border since direct microelectrode measurements (Zeuthen *et al.* 1978) showed no significant differences following piretanide, and uptake experiments (where piretanide-induced P.D. changes would have opposite effects on the movements of Na^+ and Cl^-) showed equal inhibition of both ions. It is therefore suggested that piretanide acts on a neutral coupled mechanism for the influx of these ions across the mucosal membrane. Further support comes from results of microelectrode experiments where it was found that in plaice intestine the mucosal membrane is highly permeable to Na but not to Cl, and that Cl accumulated inside the cells

remains passively distributed either in the presence of piretanide or in Na-free media (Zeuthen *et al.* 1978; Ellory *et al.* 1979).

The effects of ouabain, to inhibit Cl transport and I_{sc}, can be explained by the abolition of the Na gradient.

Although it is suggested that the major component for the Cl influx in this tissue is accounted for by a neutral coupled Na Cl influx mechanism, it has been shown that under short-circuit conditions the rate of Cl absorption is almost four times that of Na (Table 2). Similar results were found in flounder intestine (Field *et al.* 1978) where it was proposed that the process of one-for-one *transcellular* Na Cl transport would be obscured by the permselective properties of the paracellular pathway. Thus, under short-circuit conditions, much of the Na transported into the lateral intercellular spaces from the cells could recycle into the mucosal solution via Na-selective 'tight' junctions resulting in a serosa-negative transepithelial electrical potential difference and a preponderance of Cl absorption over that of Na.

This work was supported by an M.R.C. grant. M. M. P. Ramos gratefully acknowledges receipt of a Gulbenkian Foundation Studentship during these studies. We thank Stuart Dombey of Hoechst Pharmaceuticals for his help with diuretics and also Leo Pharmaceuticals and Merck, Sharp & Dohme for gifts of drugs. The expert technical assistance of Ms S. E. M. Jones is also gratefully acknowledged.

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