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Thiocyanate Transport across Fish Intestine (*Pleuronectes platessa*)

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Summary. When bathed on both sides with identical chloride-containing salines the *in vitro* preparation of the plaice intestine maintains a negative (serosa to mucosa) short-circuit current of 107 ± 11 $\mu A/cm^2$, a transepithelial potential difference of 5.5 ± 0.6 mV (serosa negative), and a mean mucosal membrane potential of -45.4 ± 0.6 mV. Under these conditions the intracellular chloride activity is 32 mM.

If chloride in the bathing media is partially, or completely substituted by thiocyanate the measured electrical parameters do not change but transepithelial flux determinations show a reduction in chloride fluxes and the presence of a significant thiocyanate flux. The addition of piretanide (10^{-4} M) reduced the short-circuit current and the mucosa-to-serosa fluxes of chloride and thiocyanate; this inhibition is similar to the effect of piretanide on chloride transport in this tissue.

The results indicate that thiocyanate is transported in this tissue via the piretanide-sensitive "chloride" pathway and are compared with the effects of thiocyanate on other tissues reported in the literature.

Key words thiocyanate · chloride · transport · intestine

Introduction

Thiocyanate has often been used as an inhibitor of anion transport in a variety of secretory epithelia. For example, it is effective in inhibiting chloride transport in fish gills (Epstein, Maetz & de Renzis, 1973; de Renzis, 1975; Maetz, 1976), in frog cornea (Zadunaisky, Lande & Hafner, 1971), and in rabbit gallbladder (Cremaschi, Hénin & Meyer, 1979). However, thiocyanate has also been reported to have a less dramatic effect on the electrical parameters in other epithelia, including fish opercular epithelium (Degnan, Karnaky & Zadunaisky, 1977), frog cornea (Akaike, 1971), and rabbit gallbladder (Cremaschi et al., 1979). Recently we reported that 40 mM thiocyanate had no effect on either short-circuit current or transepithelial potential in marine teleost intestine, although chloride influx was inhibited (Ramos & Ellory, 1981). In this preparation electrogenic Cl transport is dominant and is equivalent to the short-circuit current; it was therefore suggested that thiocyanate may be able to replace Cl and move via the Cl transporting pathway.

The present paper is directed towards investigating this proposal. Initially, electrical measurements of short-circuit current and transepithelial potential difference confirmed the earlier observation. Isotope experiments were carried out to measure transepithelial thiocyanate fluxes. Inhibition studies were performed using piretanide, as an effective anion transport inhibitor (Zeuthen, Ramos & Ellory, 1978). Finally, microelectrode experiments were used to assess the effect of thiocyanate replacement on the membrane potential measured across the mucosal membrane.

The results confirm that in the presence of 40–130 mM thiocyanate there is a significant electrogenic anion transport and a net flux of thiocyanate across the tissue.

Materials and Methods

Animals

Plaice (*Pleuronectes platessa*), weighing 100–250 g were collected in the English Channel and were kept unfed in seawater tanks at 8 °C. They were not used until at least 2 weeks after arrival in the laboratory.

Preparation of Tissue

The anterior intestine was always used. In some experiments the muscle layer was removed by dissection under a low-power microscope; thus an isolated epithelium was obtained (a stripped preparation). As far as electrical parameters and ion fluxes are concerned, this preparation behaved similarly to the intestine previously characterized (Ramos & Ellory, 1981). All experiments were carried out at room temperature (~18 °C).

Solutions

The saline solution, unless otherwise specified, contained (in mM) NaCl, 130; $MgSO_4$, 1.1; CH_3COOK, 10; $CaCl_2$, 2.5; choline bicar-

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bonate, 25; glucose, 10; alanine, 2; gassed with 95% O_2/5% CO_2, pH 7.4. Thiocyanate and isethionate replacement was always isotonic (i.e., NaCl was substituted by an equivalent amount). In the microelectrode studies, gluconate substitution was used to provide the impermeant anion. Piretanide (Hoechst Pharmaceuticals, Hounslow) was dissolved in saline and neutralized with Tris base before use.

Electrical Measurements

Transepithelial potential difference (PD), and short-circuit current (SCC), were measured in Ussing chambers (0.7 cm^2 area) as previously described (Ramos & Ellory, 1981). Calomel electrodes were used for potential measurements (the potential difference between the calomel half-cells being less than 0.2 mV), and Ag/AgCl electrodes to inject current. The electrodes were connected through saline-agar bridges. The preparation was short-circuited throughout the experiment by an automatic voltage-clamp device, which was compensated for the resistance of the bathing solutions.

Intracellular Measurements

Cl-Sensitive Microelectrodes. Single-barrelled Cl-sensitive liquid ion exchanger (LIX)-filled microelectrodes were fabricated by the method of Zeuthen, Hiam and Silver (1973). Briefly, 2-mm OD glass micropipettes with an internal fiber were prepared, having 20–40 mΩ resistance if filled with 3 M KCl. Following siliconization, the electrodes were back-filled with Corning Cl^- exchanger (Corning 477315) to a column length of about 0.5 mm, followed by 0.15 M KCl. A Ag/AgCl wire was inserted and sealed in place with silicone rubber cement (Silcoset 153). The complete electrode assembly was equilibrated for 1 hr in 200 mM KCl.

Calibration

The electrodes were initially calibrated in KCl solutions ranging from 1 to 200 mM. A calibration curve was then constructed using salines with various chloride concentrations (gluconate substitution). This calibration curve (range 135-1 mM Cl^-) was used to obtain intracellular chloride activities from the recorded electrode potential. The electrode response was linear down to 5 mM Cl^- ($r=0.998$), giving a steeper nonlinear slope at lower concentrations. Thus, concentrations recorded as lower than 5 mM were considered indistinguishable from background interference. In KCl solutions these electrodes gave a slope of 55–60 mV/decade and a resistance of 10^{10}–10^{11} Ω with time constant of $t_{\frac{1}{2}} \simeq 1$ sec.

Interference from thiocyanate ions was investigated and a selectivity coefficient of $K_{ij} \simeq 2.5$ was found, using the empirical equation given by Walker (1971). It was therefore impossible to distinguish between the Cl^- und SCN^- signals in thiocyanate saline with this system, and consequently no data for intracellular Cl^- activity is presented in the thiocyanate experiments.

Conventional Microelectrodes

Microelectrodes for measuring intracellular potential difference were pulled from Clark Electromedical Instruments GC 120F-15 borosilicate glass with internal fiber, to a tip resistance of 50–60 mΩ, and filled with 3 M KCl. Tip potential was less than 5 mV.

Measurement of Membrane Potential and Intracellular Chloride Activity

Stripped plaice intestine was mounted in a Perspex chamber (0.75 cm^2 area; 1 ml volume), independently perfused (3 ml/min) on both sides. When the perfusing solution was changed, substitution was always made only on the mucosal side, with complete replacement taking less than 30 sec. Cells were impaled by the Cl^--sensitive microelectrode from the mucosal side, with a simultaneous measurement of mucosal membrane potential (V_{mc}) using the normal 3 M KCl filled microelectrode. Both electrodes were referenced to an external bath Ag/AgCl electrode connected to the mucosal solution via a 3 M KCl-agar bridge. The mucosal bath solution was grounded by an Ag/AgCl wire.

Amplification was achieved by a pair of Analog Devices 311J operational amplifiers connected to a differential amplifier (Zeuthen et al., 1973). The output from the differential amplifier was recorded on a Bryans Southern Instruments 28 000 dual-channel pen recorder.

The criteria adopted for establishing successful penetrations were those of Duffey, Thompson, Frizell and Schultz (1979).

Flux Measurements

Thiocyanate ($KS^{14}CN$ 18.5 kBq/ml) and chloride ($Na^{36}Cl$ 7.4 kBq/ml) transfluxes (J_{13}; J_{31}) were determined in the Ussing chambers (1 ml volume on each side) under short-circuit conditions. After 60 min equilibration the isotope was added to one side of the intestine for another 30–45 min equilibration. 15-min collection periods began on the other side for $2\frac{1}{2}$–$3\frac{1}{2}$ hr.

Materials

Radioactive $KS^{14}CN$, $Na^{36}Cl$, ^3H-inulin were obtained from Radiochemical Centre, Amersham. Piretanide was a gift from Stuart Dombey, Hoechst Pharmaceuticals, Hounslow. All other chemicals were reagent grade, Sigma Chemical Co., St. Louis, Mo., or BDH, Poole, Dorset.

Students *t*-test was used for statistical analyses.

Abbreviations

SCC – short-circuit current
PD – transepithelial potential difference
J_{13} – mucosal-serosal transepithelial flux
J_{31} – serosal-mucosal transepithelial flux
J_{net} – J_{13}–J_{31}
V_{mc} – mucosal membrane potential
V_{Cl} – chloride electrochemical potential
a^i_{Cl} – intracellular chloride activity

Results

Electrical Parameters

In all our experiments (except for one fish out of 23), replacement of 40 mM chloride by thiocyanate in the bathing medium did not have any appreciable effect on the measured SCC and PD. Reducing the chloride concentration itself by 40 mM (isethionate substitution) had no effect on the electrical parameters. Figure 1 shows a recording of SCC from one experiment. It can be seen that successive increases in thiocyanate concentration up to 65 mM did not significantly alter the electrical parameters of the preparation. The highest thiocyanate concentration (130 mM) had no effect on some preparations (*see* Table 1). However, in other experiments (e.g., Fig. 1), there was a reduction in the SCC and PD. This effect was

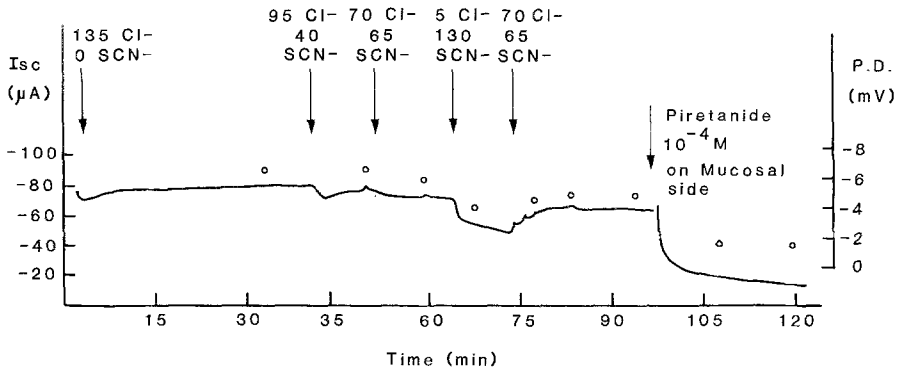

Fig. 1. The effect of successive replacement of chloride by thiocyanate on the SCC and PD of plaice intestine. The continuous line is the SCC recording, and the open circles are the open-circuit PD measurements

Table 1. Chloride and thiocyanate influx (J_{13}) in plaice intestine

Bathing medium	$J_{13}^{Cl^-}$	$J_{13}^{SCN^-}$	$\sum J_{13}^{Cl^-} J_{13}^{SCN^-}$	SCC
	($\mu mol \times cm^{-2} \times hr^{-1}$)			
A. High Cl ($n=6$)				
95 mM Cl^- 40 mM isethionate	8.2±1.6	–	8.2±1.6	3.4±1.0
95 mM Cl^- 40 mM SCN^-	4.9±0.6	3.0±0.3	7.9±0.7	2.9±0.7
95 mM Cl^- 40 mM SCN^- +Piretanide 10^{-4} M	2.6±0.2	2.0±0.2	4.6±0.3	0.1±0.1
B. Thiocyanate Ringer	($n=4$)			
0 Cl^- 130 mM SCN^-	–	7.8±0.5	7.8±0.5	1.3±0.4
+Piretanide (10^{-4} M)	–	6.4±0.2	6.4±0.2	0.2±0.1

The values are averages at steady state of the last two collection periods in each condition. *A* summarizes determinations in saline containing 95 mM Cl and 40 mM of either isethionate or thiocyanate. *B* summarizes determinations in 130 mM thiocyanate saline.

Results are given as mean±SEM (n=number of separate preparations). SCC was converted into μmol of monovalent ion being transported per cm^2/hr.

reversible upon lowering the thiocyanate concentration back to 65 mM. The figure also shows that the SCC under these conditions (70 mM Cl^-/65 mM SCN^-) is completely inhibited by 10^{-4} M piretanide.

Transepithelial Fluxes

Since replacing 40 mM chloride by thiocyanate in the saline did not change the SCC, we have determined the transflux of chloride and thiocyanate across the intestine under these conditions. Table 1 summarizes the results for chloride and thiocyanate flux experiments. Flux measurements began in chloride-isethionate saline (95 mM Cl^-/40 mM isethionate) initially measuring chloride flux (J_{13}^{Cl}). After 1 hr the isethionate medium was replaced by thiocyanate medium (40 mM), and both chloride and thiocyanate fluxes were followed. Chloride flux decreased markedly upon replacing the isethionate by thiocyanate. At steady-state the sum of the fluxes of both chloride and thiocyanate equals the chloride flux in the isethionate saline. At this point piretanide, an inhibitor of the mucosal serosal chloride transflux in this preparation (Ramos & Ellory, 1981) was added on the mucosal side at 10^{-4} M. The SCC was completely suppressed and a similar inhibition was seen in the flux (J_{13}) of both chloride and thiocyanate. The inhibition of the fluxes (J_{13}) accounts quantitatively for the reduction in the SCC. It has previously been shown that the SCC in this preparation is equivalent to the net flux of chloride. The inhibition of SCC by piretanide was due to inhibition of mucosal to serosal chloride flux – J_{13}^{Cl}, flux J_{31}^{Cl} being unaffected (Ramos & Ellory, 1981). The backflux (J_{31}) of thiocyanate in the present work, in preparations with

Table 2. Chloride and thiocyanate backflux (J_{31}) in plaice intestine

	Bathing medium	J_{31}^{Cl-}	J_{31}^{SCN-}	$\sum J_{31}^{Cl-} J_{31}^{SCN-}$	SCC
		($\mu mol \times cm^{-2} \times hr^{-1}$)			
A	High Cl ($n=5$)				
	95 mM Cl^- 40 mM isethionate	2.14 ± 0.17	–	2.14 ± 0.17	4.92 ± 0.71
	95 mM Cl^- 40 mM SCN^-	1.78 ± 0.25	1.29 ± 0.13	3.07 ± 0.28	4.83 ± 0.81
	95 mM Cl^- 40 mM SCN^- +Piretanide (10^{-4} M)	1.54 ± 0.34	1.29 ± 0.15	2.83 ± 0.37	0.18 ± 0.16

Table 3. Intracellular potential and chloride activity measurements across the mucosal membrane

	Normal saline (135 mM Cl^-)	SCN^- saline (130 mM SCN^-, 5 mM gluconate)	Gluconate saline (135 mM gluconate)
Apical membrane potential V_{mc} (mV)	-45.4 ± 0.6 ($n=31$) ($N=3$)	-45.3 ± 3.9 ($n=20$) ($N=3$)	-55.3 ± 1.2 ($n=12$) ($N=2$)
P		NS	<0.001 [a]
Chloride chemical potential V_{Cl} (mV)	$+23.0 \pm 1.4$ ($n=20$) ($N=2$)	–	$+42.6 \pm 1.6$ ($n=19$) ($N=2$)
Cl^- activity $a_i Cl$ (mM)	32		8.5

The mucosal side of the stripped intestine was perfused with the indicated saline, and a number of impalements were carried out under each experimental condition. The serosal side was perfused with normal (chloride) saline throughout. The values are mean ± SEM of the number of observations (n) and tissues (N) as indicated.

NS – not significantly different from normal saline (two tailed 't').

[a] One tailed 't'.

SCCs comparable to those used in the experiments on J_{13}, was smaller than J_{13} and was not affected by 10^{-4} M piretanide.

Table 1, section *A* summarizes data from six separate flux experiments. The values represent the steady-state fluxes, measured during the last 30 min at each experimental condition. Section *B* summarizes four determinations of thiocyanate mucosal to serosal flux where the preparations were continuously in full thiocyanate saline (130 mM). In both sets of experiments (*A* and *B*), the addition of piretanide (10^{-4} M) inhibited a substantial fraction of the anion transport, quantitatively equivalent to the reduction in SCC.

Data on the backflux (J_{31}) of chloride and thiocyanate in this preparation are presented in Table 2. As shown previously in bidirectional flux measurements (Ramos & Ellory, 1981), J_{31}^{Cl} is smaller than J_{13}^{Cl} and insensitive to piretanide. The values for J_{31}^{SCN} are similar to J_{31}^{Cl} but smaller (the ratio J^{SCN}/J^{Cl} being 0.6–0.8 for both influx and backflux). Thus, as for the chloride fluxes, J_{13}^{SCN} is substantially greater than J_{31}, indicating a piretanide-sensitive net transport of thiocyanate.

Intracellular Microelectrode Measurements

Using KCl-filled glass microelectrodes, the mean mucosal membrane potential (V_{mc}) recorded across plaice intestinal mucosa was -45.4 ± 0.6 mV in normal saline (*see* Table 3). The mucosal membrane potential was unchanged when recorded 5–20 min after mucosal substitution of thiocyanate saline. When chloride medium was resubstituted, there was again no change in the potential. Isotonic gluconate replacement on the other hand (mucosal only), hyperpolarized the membrane potential, the mean increase in V_{mc} being 15–20%. Table 3 also presents results for intracellular Cl^- activity determinations. In the control saline, the Cl^- electrode recorded an intracellular a_{Cl}^i of 32 mM, equivalent to a Cl^- concentration of about 47 mM for an activity coefficient of 0.68 (Bates, Staples & Robinson, 1970). Considering V_{mc} for passive Cl distribution, the intracellular a_{Cl}^i would have been 16 mM, equivalent to a concentration of 23 mM. When the bathing medium was substituted by gluconate saline, there was a rapid decline in intracellular a_{Cl}^i, reaching a new steady-state level around 8.5 mM in

not more than 5 min. This gluconate substitution effect was readily reversible upon readdition of control saline.

Discussion

In the present paper we have demonstrated that in fish anterior intestine where under short-circuit conditions Cl^- is the major ion carrying the current (Ramos & Ellory, 1981), thiocyanate can replace Cl^-. Thus, SCC, PD and V_{mc} were not affected by replacing some or all of the Cl^- with SCN^-. Unidirectional transepithelial flux measurements indicated that thiocyanate is about as permeable as chloride when measured in the same preparations. Piretanide, an effective inhibitor of chloride transport in this preparation (Zeuthen et al., 1978; Ramos & Ellory, 1981) also inhibited the SCC and the mucosa-to-serosa flux of thiocyanate (J_{13}^{SCN}). The microelectrode results (Table 3) also demonstrate that thiocyanate substitution has no significant effect on the mucosal membrane potential (V_{mc}). However, Cl^- replacement by the impermeant anion gluconate caused a significant and reversible hyperpolarization. (This could be interpreted in terms of the Goldman equation (Goldman, 1943) as V_{mc} tending to the potassium equilibrium potential when external Cl^- is zero.) Intracellular Cl^- activity measurements confirmed earlier studies where active Cl^- accumulation was shown (Zeuthen et al., 1978; Duffey et al., 1979). Although it was not possible to measure Cl^- activity in the presence of SCN^- with our system (*see* Methods), the fact that V_{mc} does not change in isotonic thiocyanate suggests that it may behave similarly to chloride in terms of the permeability across the mucosal membrane. Taken together, our results are consistent with thiocyanate being able completely to substitute for chloride in this preparation and be transported through the piretanide-sensitive pathway.

Thiocyanate has traditionally been used as an inhibitor of anion transport in epithelia. Two differences are apparent in surveying the results of thiocyanate effects in the literature. One concerns the inhibiting concentration of thiocyanate which is effective. In three epithelia, chloride transport in fish gill (Epstein, et al., 1973; de Renzis, 1975; Maetz, 1976), acid secretion in dog and bullfrog gastric mucosa (Davenport, 1940; Forte & Davies, 1964), and in the pancreas (Schulz, 1972), thiocyanate inhibits secretion at rather low concentrations (<4 mM). In all other epithelia, much higher concentrations were used (10–25 mM), and the results show the second discrepancy, between electrical measurements and flux determinations. Thus, when electrical parameters are assessed following thiocyanate addition, effects are small and transient (e.g., for gastric mucosa *see* Sachs et al., 1969; frog cornea, Akaike, 1971; opercular epithelium, Degnan et al., 1977; rabbit gallbladder, Cremaschi et al., 1979). In contrast, chloride flux studies indicate significant reduction by thiocyanate at the same concentration (frog cornea, Zadunaisky et al., 1971; rabbit gallbladder, Cremaschi et al., 1979). This discrepancy is accounted for by thiocyanate substituting effectively for chloride on a common transport pathway, as clearly demonstrated for fish intestine in the present work. (In fact, thiocyanate transport has previously been demonstrated for bullfrog gastric mucosa (Hogben, 1965; Sachs et al. 1969).) In rabbit ileum we found that the electrical parmeters did not change following replacement of 40 mM isethionate by thiocyanate in the bathing medium (*unpublished observations*), while Frizzell et al. (1973) reported a marked reduction in Cl^- influx caused by thiocyanate. To account for the high sensitivity of the three tissues mentioned above (fish gill, gastric mucosa, pancreas), it is necessary to consider whether in some circumstances thiocyanate has a specific inhibitory action rather than becoming a chloride substitute. It is well known that thiocyanate inhibits carbonic anhydrase at low concentrations (6×10^{-4} M, 0 °C, Davenport, 1940), and one would therefore expect tissues where carbonic anhydrase plays a major role in transport to be highly thiocyanate-sensitive. Also, it should follow that tissues showing a low thiocyanate sensitivity would be insensitive to acetazolamide, which seems in fact to be the case in the opercular epithelium (Degnan et al., 1977) and also for the frog cornea (Zadunaisky, 1978; Zadunaisky, *personal communication*). Further, evidence has been accumulated for an anion-activated ATPase, which may be involved with anion transport in gastric mucosa and fish gill. Although this aspect is controversial (Bonting, van Amelsvoort & de Pont, 1978), recent work has shown that thiocyanate is an effective inhibitor of this enzymatic activity at very low concentrations (<0.2 mM; de Renzis & Bornancin, 1977), again indicating a possible specific role for thiocyanate inhibition.

In conclusion, the present work indicates that thiocyanate can act as a chloride substitute in transporting epithelia, capable of effectively supporting active anion transport, inhibition of chloride transport simply being competitive. In a few special cases, where thiocyanate is much more potent, the mechanism of inhibition is direct, involving either carbonic anhydrase or other enzyme inhibition effects.

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