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THE BRANCHIAL CHLORIDE PUMP IN THE GOLDFISH *CARASSIUS AURATUS*: RELATIONSHIP BETWEEN Cl^-/HCO_3^- AND Cl^-/Cl^- EXCHANGES AND THE EFFECT OF THIOCYANATE

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SUMMARY

1. The effect of thiocyanate on chloride and sodium fluxes across the gill was studied in the goldfish *Carassius auratus*. At low external chloride concentrations, addition of SCN^- to the bathing solution markedly inhibited chloride influx and efflux, the net flux being reversed. SCN^- injection was without effect. SCN^- had no effect on sodium fluxes when injected or added to the external medium.

2. The inhibition of chloride influx by SCN^- was of a mixed type involving simultaneous modifications of the affinity constant of the carrier for Cl^- and of the maximal Cl^- influx. The affinity constant of the carrier for SCN^- was 10 times lower than that for Cl^-.

3. The gill of the goldfish was found to be practically impermeable to SCN^-.

4. In the presence of external SCN^-, the Cl^-/HCO_3^- exchange was reversed: Cl^- was lost against HCO_3^- which is absorbed. This suggests an obligatory exchange.

5. Exchange diffusion for chloride was also demonstrated.

6. A kinetic model is proposed to explain chloride and bicarbonate transport across the gill of *Carassius auratus*.

INTRODUCTION

Branchial absorption of chloride from the external medium in the presence of an impermeant co-ion has been described in a number of freshwater animals (see review by Maetz, 1974*a*). To maintain electroneutrality between the external and internal media such an absorption requires an exchange with an endogenous ion of the same charge. Krogh (1939) first suggested the existence of a Cl^-/HCO_3^- exchange mechanism. This hypothesis was indirectly verified in the goldfish by Maetz & Garcia-Romeu (1964) and has been confirmed directly in the same species. An apparent correlation between the net absorption flux of chloride and the net excretion flux of base (probably HCO_3^-) has been observed by de Renzis & Maetz (1973).

Since thiocyanate has inhibitory properties on halide transport in tissues such as the thyroid (Wolff, 1964), the stomach (Forte, 1972) and the cornea (Zadunaisky, Lande & Hafner, 1971), its effects have been studied on the fish gill (Epstein, Maetz &

de Renzis, 1973; Kerstetter & Kirschner, 1974). In the eel adapted to sea water, the chloride excretion pump was markedly inhibited by SCN^- injection. In contrast, the sodium efflux was not affected. In the goldfish, Krogh (1938) already noted that addition of SCN^- to the external medium was followed by a loss of chloride. Inhibition of chloride absorption was thought to be the result of a modification of the permeability of the gill. Epstein *et al.* (1973) demonstrated with the aid of isotopic techniques that in *Carassius*, the influx and net absorption flux of Cl^- were strongly inhibited by the external addition of SCN^- while the Na^+ netflux was not changed. Kerstetter & Kirschner (1974) confirmed in *Salmo gairdneri* that SCN^- added to the external medium markedly inhibits Cl^- influx. Na^+ influx was unaffected. SCN^-, injected into the fish, does not alter these fluxes.

In the present work, the mode of action of SCN^- on the unidirectional chloride fluxes and on Cl^-/HCO_3^- exchange has been studied. In particular the type of inhibition of the chloride influx by SCN^- has been investigated. Moreover, the permeability of the gill to this inhibitor has been determined in an attempt to verify whether SCN^- was able to substitute for Cl^- in the pumping mechanism.

MATERIALS AND METHODS

The experiments were performed on the goldfish *Carassius auratus* obtained from a hatchery near Paris. The animals weighed 75–150 g and were maintained, unfed, in aquaria in running fresh water (9–18 °C).

Preparation of animals

Most of the experiments were done on fish taken directly from freshwater aquaria (Na^+ = 105 μEquiv/l, Cl^- = 40 μEquiv/l, Ca^{2+} = 1340 μEquiv/l, HCO_3^- = 800 μEquiv/l). Experiments to study the effect of SCN^- on Cl^-/HCO_3^- exchange, however, were made on animals previously maintained for 3–4 weeks in an unbuffered Na_2SO_4 solution (0·5 mM); this pretreatment was designed to augment their capacity to absorb chloride (Garcia-Romeu & Maetz, 1964; de Renzis & Maetz, 1973). On the day before the experiments a catheter was inserted into the urinary papilla of each fish. The animals were then placed in individual aquaria in open circuit and aerated; the external medium was similar to that used during adaptation (fresh water or Na_2SO_4 solution). In the 2 h preceding an experiment the external medium was replaced by 30 l of deionized water buffered (pH 7·4) with a mixture of imidazole (2 mM) – sulphuric acid (0·07 mM). The aquaria were then placed in closed circuit which included in series: a pump (200 ml/min) ensuring good mixing, a cooling unit which maintained a constant temperature of 16 °C and a β-detector connected to an automatic system measuring radioactivity (Tanguy, 1970).

Flux measurement

The influxes of Cl^-, Na^+ and SCN^- were measured during different experiments by following the disappearance of radioactivity from the external medium as $^{36}Cl^-$, $^{24}Na^+$ (CEN, Saclay) or $S^{14}CN^-$ (Radiochemical Centre, Amersham). The methods of calculation used have been described previously for sodium fluxes by Maetz (1956). Since the experiments were of short duration the radioactive 'backflux' has not been taken into account for the influx calculation. The net Cl^- and Na^+ fluxes

were calculated in all experiments by determining the concentration variations of these two ions in the external medium. Chloride was measured by amperometric titration (Aminco–Cotlove), sodium by flame photometer (Eppendorf). It should be noted that, in the range of concentrations used, SCN^- was measured, like Cl^-, by amperometric titration. To calculate the chloride concentrations in those samples containing both Cl^- and SCN^- the calculated value for SCN^- concentration in the bath was deducted from the value found by titration. As shown below, SCN^- is impermeant, hence its concentration remained constant during the experiments. The error involved in this method intervenes only in the influx calculation and then to a negligible extent.

Effluxes were measured for each period by taking the difference between influx and net flux. In experiments designed to study the action of thiocyanate on Cl^-/HCO_3^- exchange the net fluxes of base and total ammonium have likewise been calculated from the variations in titratable alkalinity and in total ammonium in the bath.

The calculation of base actually excreted against chloride has been described previously (de Renzis & Maetz, 1973). Titratable alkalinity was determined with an automatic titrator (Tacussel). Ammonium was measured with an autoanalyser (Technicon).

Branchial permeability to thiocyanate

To measure the permeability of the gill to SCN^- the aquarium was filled with imidazole sulphate solution (pH 7·4) containing $KS^{14}CN$ (200 μM, approx. 0·025 μCi/ml). Samples of the external medium and also of the solution used to fill the aquaria were taken for counting by liquid scintillation (SL 40 Intertechnique). At the end of the experiment blood was taken from the caudal artery. The radioactivity of 1 ml plasma was determined using Bray's solution after solubilization with Soluene 350 (Packard).

Study of the action of thiocyanate on Cl^-/HCO_3^- exchange

This study was made on pretreated animals (see above). Branchial flux measurements were made in the absence of sodium in the external medium to avoid the possible liberation of H^+ ions, exchanged against Na^+ (Maetz, 1973) and their combination with HCO_3^- ions, exchanged against Cl^-, to give CO_2 which escapes. Radioactivity was added at the start of the experiment in the form of [^{36}Cl]choline. SCN^-, as KSCN, was added after a 2 h control period and its effects followed during a further 2 h. Samples of the external medium were taken every 20 min for determinations of chloride and titratable alkalinity.

All flux measurements were carried out after homogenization periods of 20–30 min. Fluxes are expressed as μEquiv/h.100 g.

Determination of chloride space

The distribution space for chloride was measured using one of the techniques described by Mayer & Nibelle (1969). The isotope is added to the external medium and the distribution volume is calculated from the relationship

$$V_{\text{int}} = \frac{Q_{0\,\text{ext}} - Q_{1\,\text{ext}}}{[Cl^*_{\text{int}}]_{t_1}},$$

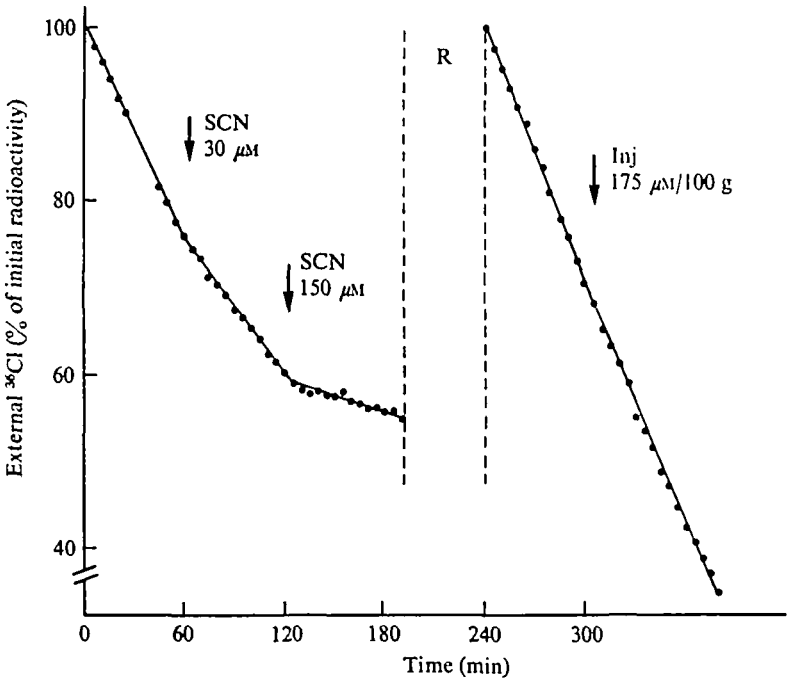

Fig. 1. Effect of external and internal addition of thiocyanate on chloride absorption by *Carassius auratus*. Reversibility of action. At arrows: addition or injection (inj) of SCN^-. The values given are final concentrations of inhibitor. In this experiment the initial external total NaCl concentration was 490 μM. Note changes of slope indicating changes in the disappearance rate of external ^{36}Cl; R, rinsing of the external medium.

where Q_0 and Q_1 are the quantities of isotope in the external medium at the times t_0 and t_1 respectively and $[Cl^*_{int}]_{t_1}$ the plasma concentration of radioactive chloride at t_1.

Blood was drawn from the caudal blood vessels by means of a heparinized syringe.

Potential measurement

In some experiments the potential difference between the intraperitoneal cavity and the external medium was measured on unanaesthetized fish by the technique described by Maetz (1974*b*).

RESULTS

Effects of thiocyanate on chloride fluxes

Fig. 1 shows the course of a typical experiment in which the action of SCN^- was studied on the external surface of the gill, and then, after rinsing, on the internal surface. Fig. 1 shows that SCN^- placed in the external medium, that is, in the compartment from which the chloride is pumped, strongly inhibits chloride influx, whereas injection remains without effect. The degree of inhibition is dose-dependent: for a Cl^-/SCN^- ratio equal to 16, the inhibition is 20%, whereas for a value close to 3 it reaches 80%. Finally the figure shows that the effect of the inhibitor was completely abolished after 60 min rinsing.

Table 1 summarizes the effects of SCN^- on the influx, efflux and net flux

Table 1. *Effects of thiocyanate on chloride fluxes of* Carassius auratus

	$[Cl]_{ext}$	f_{in}	f_{net}	f_{out}
Control ($n=21$)	365	$48{\cdot}2 \pm 3{\cdot}7$	$+12{\cdot}8 \pm 4{\cdot}1$	$35{\cdot}4 \pm 3{\cdot}9$
SCN^-_{ext} ($n=21$)	359	$10{\cdot}5 \pm 1{\cdot}9^{***}$	$-9{\cdot}8 \pm 3{\cdot}3^{***}$	$20{\cdot}3 \pm 3{\cdot}7^{**}$
Rinse ($n=4$)	440	$51{\cdot}1 \pm 6{\cdot}3$	$+9{\cdot}1 \pm 13{\cdot}6$	$42{\cdot}0 \pm 11{\cdot}7$
SCN^-_{int} ($n=3$)	417	$54{\cdot}5 \pm 10{\cdot}2$	$-4{\cdot}6 \pm 29{\cdot}6$	$59{\cdot}1 \pm 20{\cdot}8$

External thiocyanate concentration: 150 μM. Injections: 150 μM/100 g. Fluxes in μEquiv/h. 100 g. Chloride concentrations in μM. As in other tables, means ± S.E. are given.
*** $P < 0{\cdot}001$; ** $P < 0{\cdot}01$.

Table 2. *Absence of effect of thiocyanate on sodium fluxes of* Carassius auratus *measured in a NaCl solution*

	$[Na]_{ext}$	f_{in}	f_{net}	f_{out}
Control	525	$90{\cdot}1 \pm 6{\cdot}0$	$+47{\cdot}6 \pm 11{\cdot}3$	$42{\cdot}5 \pm 10{\cdot}9$
SCN^-_{ext}	571	$90{\cdot}2 \pm 6{\cdot}4$	$+59{\cdot}6 \pm 4{\cdot}2$	$30{\cdot}6 \pm 6{\cdot}8$
Rinse	461	$89{\cdot}5 \pm 13{\cdot}2$	$+47{\cdot}3 \pm 5{\cdot}3$	$42{\cdot}2 \pm 14{\cdot}7$
SCN^-_{int}	571	$83{\cdot}4 \pm 12{\cdot}5$	$+13{\cdot}8 \pm 28{\cdot}4$	$69{\cdot}6 \pm 38{\cdot}3$

Units as in Table 1; $n=4$.

chloride. The data were pooled from experiments made either with choline chloride or with sodium chloride in the external medium. After addition of SCN^-, the mean external chloride concentration being approximately 360 μM, both influx and efflux are significantly reduced, by 80% and by 40% respectively. The net flux changes from positive to negative. Fluxes measured after a rinse period of 45–60 min are approximately equal to those measured during the control period. Intraperitoneal injection of SCN^- produces no significant modification of the fluxes.

Effect of thiocyanate on sodium fluxes

Table 2 shows that neither the addition of SCN^- to the external medium nor its injection into the intraperitoneal cavity produce any significant variations in the sodium fluxes measured in NaCl solution.

Therefore SCN^- specifically inhibits chloride exchange across the gill of the goldfish.

Measurement of thiocyanate influx

Since SCN^- is very close to chloride in the lyotropic series it is possible that the inhibition might be due to substitution of SCN^- for Cl^- and thus to SCN^- absorption instead of Cl^-. Thus to detect any competition between the absorption of these two ions SCN^- influx was measured in the absence and then in the presence of chloride in the external medium.

Fig. 2 illustrates the contrast between the rates of absorption of $^{36}Cl^-$ and $S^{14}CN^-$ from the external medium by two goldfish. After 4 h, 65% of $^{36}Cl^-$ had disappeared whereas the $S^{14}CN^-$ concentration remained practically unaltered.

Results from a large number of experiments confirms those given above: during a 1 h period in the absence of external chloride, variation in $S^{14}CN^-$ radioactivity is $-0{\cdot}36 \pm 0{\cdot}31$% of the initial value ($n = 16$). The same calculation made for experiments in the presence of external chloride gives a variation of $+0{\cdot}16 \pm 0{\cdot}08$% in the

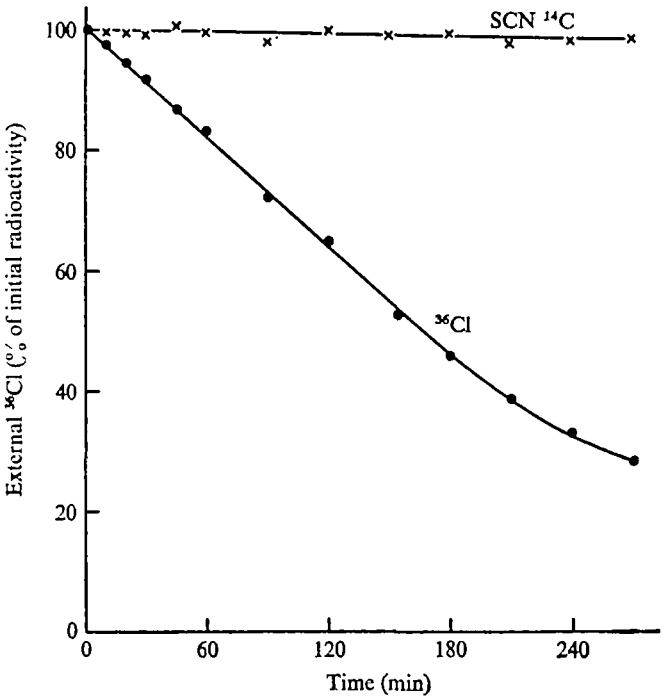

Fig. 2. Variations as a function of time of $S^{14}CN^-$ and $^{36}Cl^-$ concentrations in the external medium measured in experiments involving two different goldfish. Initial total SCN^- and Cl^- concentrations: 200 μM.

initial radioactivity per hour ($n = 4$). The two values are not significantly different from zero and demonstrate an almost total impermeability of the goldfish gill to SCN^-.

To evaluate with more precision the small influx of SCN^-, the appearance of $S^{14}CN^-$ in the plasma was measured at the end of 10 of the above mentioned experiments. For this calculation the SCN^- space was assumed to be equal to the chloride space ($32{\cdot}3 \pm 0{\cdot}56\%$; $n = 5$). The value obtained for the thiocyanate influx is $0{\cdot}52 \pm 0{\cdot}09$ μM/h.100 g. Chloride influx measured in an external ion concentration of 200 μM is $38{\cdot}8 \pm 3{\cdot}69$ μM/h.100 g ($n = 12$) – that is, 75-fold greater than the SCN^- influx.

Thus SCN^- is practically impermeable and its action on the chloride fluxes is not due to substitution of SCN^- for Cl^- in the transport mechanism.

Inhibition of chloride influx by thiocyanate

Chloride influx was measured at different levels of external chloride and in the absence or presence of SCN^- at two different concentrations: 15 and 60 μM. The procedure for this type of experiment has already been described (de Renzis & Maetz, 1973). The results are presented in Table 3.

Values for chloride influx at different ion concentrations are plotted on curves corresponding to hyperbolic functions described by an equation analogous to that of Michaelis–Menten (de Renzis & Maetz, 1973). The results have been analysed to determine F_{in}max, the maximal value for influx, and the affinity constant of th

Table 3. *Variations of chloride influx as a function of external concentration in* Carassius auratus, *in the absence (control) and in the presence of* 15 *or* 60 *μM of external SCN⁻*

Range of $[Cl]_{ext}$	Control		$[SCN^-]_{ext}=15$ μM		$[SCN^-]_{ext}=60$ μM	
	$[Cl]_{ext}$	f_{in}	$[Cl]_{ext}$	f_{in}	$[Cl]_{ext}$	f_{in}
0–100	52·4±7·6	17·5±2·8	59·9±8·2	9·4±2·0	—	—
	$n=17$		$n=10$			
100–300	186·0±12·3	31·9±2·8	136·2±5·1	16·4±2·2	145·5±23·9	8·0±1·4
	$n=24$		$n=5$		$n=9$	
300–500	437·2±12·9	49·7±4·9	398·6±11·2	29·5±5·2	467·7±27·4	18·7±1·3
	$n=13$		$n=5$		$n=4$	
500–1000	890·2±36·0	40·1±3·8	649·2±15·4	28·3±4·0	801·5±39·0	20·0±1·9
	$n=12$		$n=5$		$n=4$	

Units as in Table 1.

carrier for Cl^- in the absence of SCN^-: K_m or in the presence of SCN^-: K_x as well as the affinity constant of the carrier for SCN^-: K_i.

The influx F_{in}, as a function of chloride concentration (S) in the presence or absence of SCN^-, was plotted graphically, the line being fitted by eye. F_{in}max, K_m, K_x were thus obtained empirically.

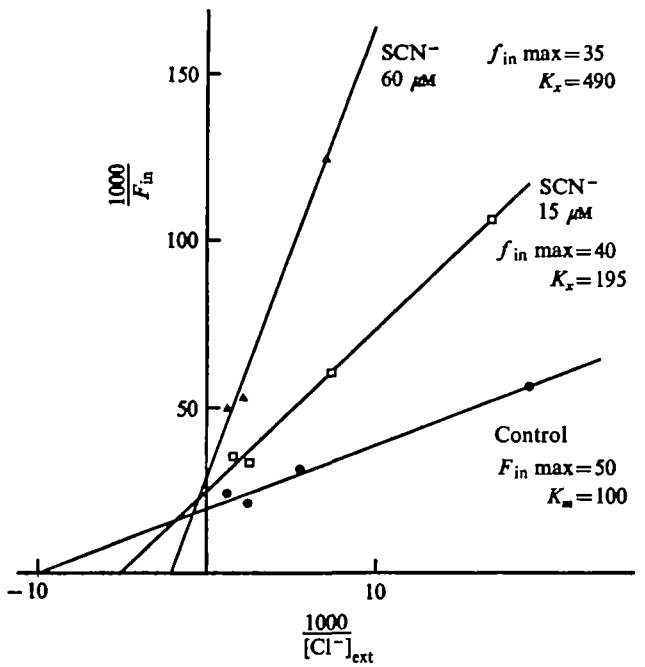

Fig. 3. Lineweaver–Burk plot showing the effects of two different concentrations of thiocyanate on chloride influx. Fluxes are measured in NaCl solutions. Thiocyanate is added as Na SCN. Maximal influxes (F_{in}max or f_{in}max) in μEquiv/h. 100 g; Km or Kx) in μEquiv/l.

The three other techniques involved plots of F_{in} *v.* F_{in}/S, S/F_{in} *v.* S and $1/F_{in}$ *v.* $1/S$, as recommended by Dowd & Riggs (1965) for enzyme kinetics. The values obtained for the various parameters are very close to those given in Fig. 3, which makes use of the Lineweaver–Burk plot.

These values show a simultaneous variation of the maximal influx and affinity constant of the carrier for chloride and suggest that the inhibition of chloride influx by SCN^- is of a mixed type. To verify whether inhibition by SCN^- was not of a simple competitive type, the maximal Cl^- influxes attained for $[Cl]_{ext} > 300$ μM were compared in control fish and in fish with 15 or 60 μM SCN^- in the external medium. These fluxes were respectively $45{\cdot}1 \pm 3{\cdot}3$ ($n = 25$), $28{\cdot}9 \pm 3{\cdot}1$ ($n = 10$) and $19{\cdot}4 \pm 1{\cdot}1$ μEquiv/h.100 g ($n = 8$). Statistical analysis showed that the difference between the two first means was highly significant ($P < 0{\cdot}01$). The difference between the means of the SCN^- treated fish was also significant ($P < 0{\cdot}02$).

Determination of the affinity constant of the carrier for thiocyanate (K_i) poses a few problems in the case of mixed-type inhibition. A value for this constant has been calculated from the formulae given by Dixon & Webb (1964, p. 327).

$$\frac{1}{K_x} = \frac{1 + \dfrac{iK_m}{K_i K'_m}}{K_m\left(1 + \dfrac{i}{K_i}\right)} \quad \text{and} \quad \frac{1}{f_{\text{in}}\max} = \frac{1 + \dfrac{iK_m}{K_i K'_m}}{F_{\text{in}}\max}.$$

Taking the ratio of these two equations gives:

$$\frac{K_x}{f_{\text{in}}\max} = \frac{K_m\left(1 + \dfrac{i}{K_i}\right)}{F_{\text{in}}\max}.$$

The value of K_i has been calculated by substituting for K_x, $f_{\text{in}}\max$, K_m and $F_{\text{in}}\max$, their value at the two thiocyanate concentrations (i) and has been found to equal 10 μM.

Effect of thiocyanate on Cl^-/HCO_3^- exchange

De Renzis & Maetz (1973) have shown directly the existence of a Cl^-/HCO_3^- exchange in the goldfish. The effect of SCN^- on this exchange was investigated. Fig. 4 shows the results of a typical experiment. Variations in chloride concentration and titratable alkalinity were followed in the external medium as a function of time before and after the addition of external SCN^- (190 μM). The action of SCN^- was characterized by an inversion of the net chloride flux, like that already shown in Table 1, and similarly by an inversion of the net flux of base. This result is confirmed by the data obtained in 7 experiments of the type presented in Fig. 4 and illustrated in Fig. 5. The tentative regression line in Fig. 5 was computed from the experimental data before and after SCN^- inhibition. The slope of this line, $0{\cdot}67 \pm 0{\cdot}06$, is equal to that found previously (De Renzis & Maetz, 1973) for control fish (i.e. $0{\cdot}71 \pm 0{\cdot}02$) and suggests an exchange of $4Cl^-$ against $3HCO_3^-$. In view of the poor precision of the data, especially those obtained after SCN^- inhibition, one cannot infer that the stoichiometry before and after SCN^- is the same. It may be noted, however, that the value of the ratio between the mean of the net fluxes of base and that of the net fluxes of chloride before the action of SCN^-: 0·77 was close to that calculated with the values obtained after the action of the inhibitor: 0·72. These values also agree with that of the slope of the regression line in Fig. 5.

The inversion of the Cl^- and HCO_3^- fluxes is accompanied by their reduction: the net chloride fluxes are between 0 and +40 normally and between 0 and −20

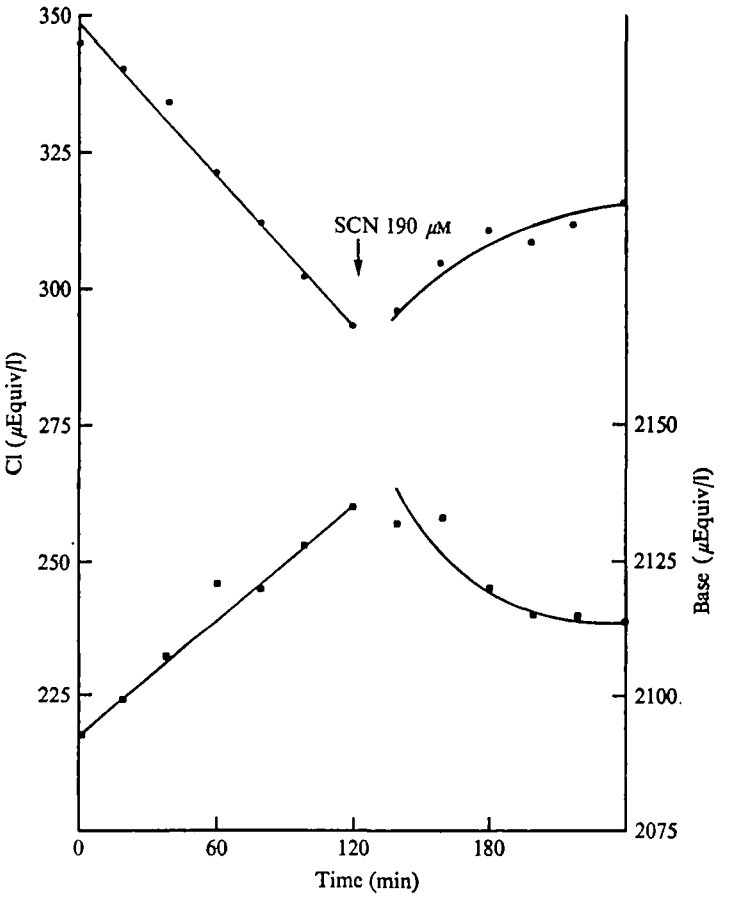

Fig. 4. Comparison between the variations of external concentration of total Cl^- and base corrected for ammonia (see text) in a typical experiment involving *Carassius auratus* before and after thiocyanate addition (at arrow: final concentration given). ●, Cl^- concentration; ■, base concentration.

after the action of SCN^-. Corresponding ranges for the net fluxes of base are 0, −30 and 0, +15 respectively.

DISCUSSION

Mode of action of thiocyanate on branchial chloride and sodium fluxes

Fig. 1 shows that the addition of SCN^- to the external medium is characterized by a rapid and reversible action on chloride influx. Epstein *et al.* (1973) have observed that the net sodium flux was not significantly altered by the action of SCN^- applied to the external or internal media of the goldfish. Results from the analysis of unidirectional sodium fluxes (Table 2) show that SCN acts neither on influx nor efflux. This confirms the frequently described independence of the absorption mechanisms for chloride and sodium and demonstrates that the action of thiocyanate is specific. Similar results were reported for the trout by Kerstetter & Kirschner (1974).

SCN^- is an inhibitor of carbonic anhydrase (Davenport, 1940). Its action in the present experiments cannot be explained in terms of inhibition of this enzyme.

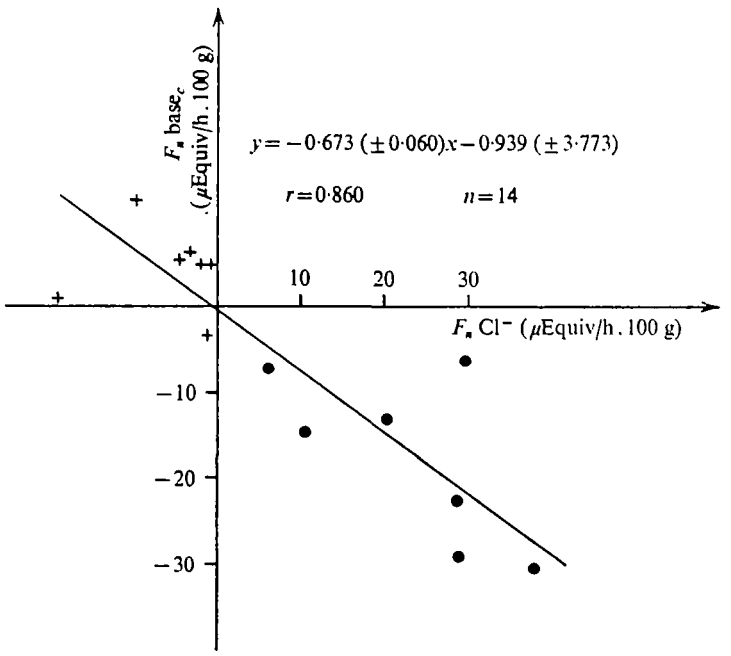

Fig. 5. Correlation between the net flux of base corrected for the net flux of ammonia and chloride uptake from a choline chloride solution by *Carassius auratus*, before (●) and after (+) addition of thiocyanate.

Maetz (1956) has shown that injection of acetazolamide, an inhibitor of carbonic anhydrase markedly diminishes the sodium influx in the goldfish. In fact, neither the injection nor the addition of SCN^- to the external medium modify the sodium fluxes. Maetz & Garcia-Romeu (1964) have found evidence that acetazolamide also produces a marked inhibition of Cl^- influx which suggests that it is the rate of production of HCO_3^- ions by the branchial cell which is the limiting factor for the HCO_3^-/Cl^- exchange mechanism and that it is indeed this ion rather than OH^- which is the endogenous ion exchanged. The current observation that the injection of thiocyanate does not modify the chloride influx therefore confirms again that SCN^-, in the doses used, remains inactive on the branchial carbonic anhydrase.

The inhibition of chloride fluxes by the addition of SCN^- to the external medium, as well as the effects of injected acetazolamide, suggest that the mechanism responsible for chloride absorption is situated on the apical face of the branchial cells, the surface in contact with the medium from which the chloride is pumped. This suggestion agrees with the results reported for trout gill by Kerstetter & Kirschner (1974), which show an inhibition of chloride influx by externally applied thiocyanate. It also agrees with the observations of Zadunaisky *et al.* (1971), who found an inhibition of the chloride flux (aqueous–lachrymal) across the cornea of *Rana catesbiana* following addition of SCN^- to the aqueous side. Similarly, inhibition of the iodide concentrating mechanism in the thyroid is observed after the addition of SCN^- to the blood (Wolff, 1964). Also, in the gastric mucosa of the toad, transport of H^+ ions linked to active chloride transport in the serosal–mucosal direction, is inhibited when SCN^- is added to the serosal side (Durbin, 1964).

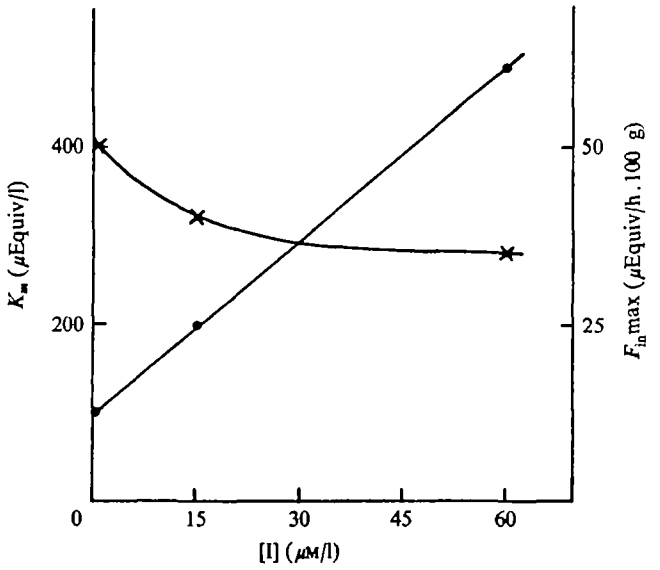

Fig. 6. Variations of the affinity constant of the Cl^- carrier (●) and of the maximal chloride influx (×) as a function of external thiocyanate concentration.

Type of inhibition of chloride influx by thiocyanate

After addition of external SCN^- a simultaneous diminution in the affinity of the chloride carrier and a reduction in the maximal chloride influx occurs. Fig. 6 shows the simultaneous change in the affinity constant and the maximal influx as functions of the concentration of the inhibitor in the external medium. The affinity constant varies in a linear manner as might be expected in the presence of an inhibition of competitive type. Inhibition of the *maximal* influx (for high $[Cl]_{ext}$) seems to tend towards a maximum at about 40% inhibition.

This unexpectedly complex type of inhibition suggests that SCN^- may possibly have more than a single binding site: one where Cl^- and SCN^- compete for the same site with a resulting increase of the K_m, and another where binding of SCN^- inhibits the breakdown of the carrier–Cl^-–SCN^- complex with a resulting decrease of the maximal rate of influx.

Enzyme kinetic studies made on the thyroid (Wollman, 1956) and on the gastric mucosa (Durbin, 1964) have shown that thiocyanate is a competitive inhibitor of iodide fluxes in the former and of H^+ fluxes, which are linked to chloride, in the latter. However, it should be noted that Durbin observed a progressive fall in acid secretion in the presence of SCN^- which he suggested might be due to the inhibitor having more than one site of action.

Nevertheless, from the value for K_i (10 μM) found in the present work it seems that the affinity of the carrier for thiocyanate might be about ten times greater than that for chloride although SCN^- is not transported. It should be noted that, for the gastric mucosa, Durbin found a factor of 14 between the affinity of the H^+ transport for Cl^- and for SCN^-.

Action of thiocyanate on Cl^-/HCO_3^- exchange

The present investigation clearly demonstrates the obligatory nature of this exchange. In effect, inhibition of the chloride flux by the addition of external SCN^- involves a simultaneous inversion of the net flux of Cl^- and a reversal of the net flux of HCO_3^-. Since the ratios between the net fluxes of these two ions are the same before and after the action of SCN^-, it may be suggested that the same mechanism allows for the entry or exit of Cl^- and for the entry and exit of HCO_3^-. Furthermore (Table 1), in the presence of SCN^- in the external medium there is a simultaneous reduction of chloride influx and efflux. These various phenomena suggest that an exchange diffusion mechanism for chloride exists across the goldfish gill.

Evidence for an exchange diffusion of chloride

An exchange diffusion for chloride in the goldfish is implied but not proved by the observed correlation between efflux and external ion concentration. Such a correlation is found in fish kept in fresh water or deionized water in which the pumping mechanism is relatively slow and in fish maintained in sodium sulphate in which the mechanism is activated.

From the data reported by de Renzis & Maetz (1973), efflux values may be plotted against external chloride concentration. They yield curves corresponding to a hyperbolic function of the Michaelis–Menten type:

$$f_{out} = \frac{F_{out}\max . C}{K_m\,\text{out} + C} + A,$$

where f_{out} is the efflux, $F_{out}\max$ the maximal efflux, C the external chloride concentration, K_m out the concentration of chloride for which $f_{out} = \frac{1}{2}F_{out}\max + A$, A the value of f_{out} when the external chloride concentration is equal to zero.

Table 4 shows the values for maximal influx ($F_{in}\max$) and for K_m (K_m in) reported in 1973 and the values corresponding to the maximal efflux ($F_{out}\max$), the K_m (K_m out) and the losses (A).

It should be noted that in every instance the values for K_m out are higher than those for K_m in. Nevertheless, one cannot infer from this comparison that the affinity of the carrier is weaker when it secures the passage of chloride from interior to exterior. In fact it would be necessary to know the value of the intracellular Cl^- concentration. If this concentration exceeds the external one (which is probable since the presence of an active pump mechanism on the apical cell surface has been shown) the values of K_m out given are underestimated. The chloride carrier affinity is thus probably lower when it functions in transport directed from the interior to the exterior than in the reverse direction.

Another argument in favour of chloride exchange diffusion in the goldfish is the existence of an excellent correlation between the ion influx and efflux measured in the presence of varied concentrations of choline or sodium chloride on animals previously maintained in running fresh water ($f_{out} = 0{\cdot}498\ (\pm 0{\cdot}009)\ f_{in} + 6{\cdot}611\ (\pm 1{\cdot}538)$; $n = 112$, $r = 0{\cdot}514$; $P < 0{\cdot}001$). This correlation has been obtained by pooling flux measurements from the present work and the preceding study (de Renzis & Maetz, 1973).

Table 4. *Characteristics of the Cl^- carrier in the goldfish*

Adaptations medium	Fluxes measured in:	F_{in} max	K_m in	F_{out} max	A	K_m out
Tap water	NaCl	55	75	30	5	175
	Cl-chol	45	75	25	5	175
Sodium sulphate	NaCl	150	40	75	10	75
	Cl-chol	115	40	65	10	75
Deionized water	NaCl	45	40	30	5	100
	Cl-chol	25	40	25	5	100

Maximal influxes and outfluxes (F_{in} max, F_{out} max); affinity constant for the transport of chloride from the exterior to the interior of the cell (K_m in); apparent affinity constant for the reverse transport (K_m out); values of the outflux when $[Cl]_{ext} = 0$ (A). Effect of different pretreatments and of substitution of sodium by an impermeant ion. For units, see Table 1.

A supplementary argument in favour of exchange diffusion lies in the finding that the same correlation between influx and efflux persists in the presence of external thiocyanate ($f_{out} = 0{\cdot}514\ (\pm 0{\cdot}011)\ f_{in} + 10{\cdot}459\ (\pm 1{\cdot}414)$; $n = 108$; $r = 0{\cdot}465$; $P < 0{\cdot}001$). Neither the slope of this regression line nor the intercept with the Y axis differ significantly from those given above.

Measurements of the transbranchial potential made by Maetz (1974*b*, and personal communication) show that in no instance do the potential variations resulting from changes in external chloride concentration account for the accompanying changes in efflux. In effect the potential difference ($\phi_{int} - \phi_{ext}$) measured in the absence of external chloride between the intraperitoneal cavity and the external medium (in deionized water – imidazole/H_2SO_4), is equal to $-46{\cdot}2 \pm 2{\cdot}81$ mV ($n = 26$). In the presence of 1 mM external NaCl the p.d. is $-33{\cdot}3 \pm 1{\cdot}52$ mV ($n = 30$). In 1 mM choline chloride its value is $-34{\cdot}9 \pm 1{\cdot}86$ ($n = 7$). Knowing these potentials the Goldman equation (1943) permits the calculation of the theoretical ratios between the efflux measured in the absence of chloride ($f_{out}0$) and those measured in the presence of 1 mM-NaCl or choline chloride ($f_{out}1$), thus:

$$\frac{f_{out}^{0}}{f_{out}^{1}} = \frac{(\phi_{int} - \phi_{ext})^{0}}{(\phi_{int} - \phi_{ext})^{1}} \quad \frac{\exp\left(\frac{ZF(\phi_{int} - \phi_{ext})^{0}}{RT}\right)}{\exp\left(\frac{ZF(\phi_{int} - \phi_{ext})^{0}}{RT}\right) - 1} \quad \frac{\exp\left(\frac{ZF(\phi_{int} - \phi_{ext})^{1}}{RT}\right) - 1}{\exp\left(\frac{ZF(\phi_{int} - \phi_{ext})^{1}}{RT}\right)},$$

where R, T and F are the classical thermodynamic constants giving the value of RT/F equal to 25 mV at the experimental temperature (16 °C) and Z is the ionic charge. The two values found for the ratio are 1·21 for external NaCl and 1·18 choline chloride respectively. From these results it appears that the increase in external chloride concentration should be accompanied by a diminution of the efflux and not by an augmentation.

The effect of variations in the potential difference on the chloride efflux under the influence of thiocyanate has likewise been estimated. In the presence of 550 μM choline chloride the p.d. for four fish had been found to equal $-29{\cdot}5 \pm 5{\cdot}3$ mV. After addition of KSCN (250 μM) to the external medium it stabilized at $-26{\cdot}7 \pm$ 3 mV. The ratio $f_{out}SCN^-/f_{out}$ control calculated according to the Goldman

equation is equal to $0{\cdot}955 \pm 0{\cdot}009$; that is, the addition of SCN^- should theoretically be accompanied by a diminution of $4{\cdot}5 \pm 0{\cdot}9\%$ of the efflux. Now the effective variation observed from the experiments is 47·7%, close to that given for a larger number of measurements (40%, Table 1). The variations in the transbranchial potential under the action of thiocyanate, or those produced by the addition of external chloride do not, therefore, in any instance, explain the changes in efflux.

A coupling between the influx and efflux could be explained by the phenomenon of 'back-transport' described by Kirschner (1955): a membrane carrier with a certain number of specific binding sites for ions allows the passage of ions from the external to internal surface where it releases them, returning empty to the external surface. Efflux takes place by free diffusion down an electrochemical gradient. When the external concentration becomes low, some of the transport sites which are free are then apt to pick up ions coming from the internal medium and therefore to return them to the interior of the cell. Thus, when the external ion concentration is high the efflux of this ion is also high, and when the external concentration is low the efflux is reduced. An increase in the activity or the number of transport sites is expressed as an increase in influx and a decrease in efflux.

The present observations do not suggest the existence of such a mechanism in the cells of the goldfish gill. In fact, earlier results (de Renzis & Maetz, 1973) show that adaptations (freshwater, deionized water, Na_2SO_4 solution) which were intended to vary the activity or number of chloride transport sites are expressed as parallel variations in influx and efflux. As we have seen above, such variations cannot be explained in terms of 'back transport'.

On the other hand, in the presence of high external chloride, that is, when all the Cl^- binding sites are occupied, addition of external SCN^- reduced influx and efflux simultaneously. If these two fluxes were linked via 'back-transport', the efflux would not have been modified.

These results, then, show that 'back-transport' does not play any major role in chloride exchanges and confirm the existence of a true exchange diffusion mechanism for chloride in the goldfish gill.

The possibility of an exchange diffusion process for Cl^- has been suggested before by Shaw (1960) for the crayfish, and rigorously demonstrated by Stobbart (1974) for the mosquito larvae. In the trout, however, Kerstetter & Kirschner (1972) reported no augmentation in the Cl^- efflux as the external Cl^- concentration was augmented from 300 μM to 1 mM and the influx increased significantly.

A model for the chloride pump

The model presented in Fig. 7 is derived from that proposed by Stobbart (1974) for the Cl^- pump associated with a Cl^-/HCO_3^- exchange and a Cl^-/Cl^- exchange in the anal papillae of *Aedes aegypti*. It has been modified to fit in the reported reversal of Cl^-/HCO_3^- exchange as a result of SCN^- inhibition. Fig. 7 suggests the possibility of a HCO_3^- transport from the external medium to the cell interior. Such a model would explain the earlier report by Maetz & Garcia-Romeu (1964) showing that addition of HCO_3^- to the external medium produces in the goldfish an inhibition of Cl^- transport. It is therefore probable that external HCO_3^- and Cl^- ions compete for the Cl^- carrier.

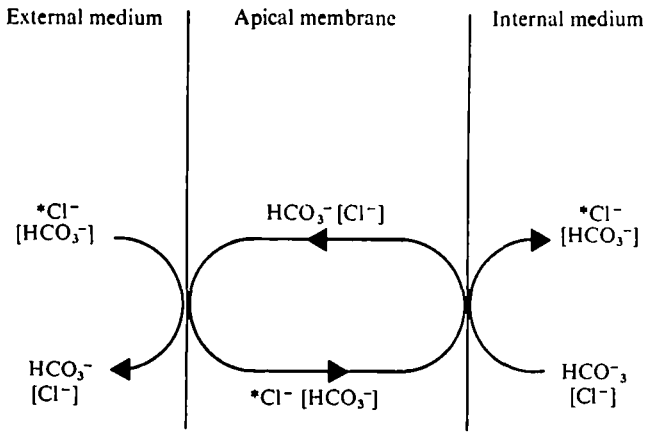

Fig. 7. A kinetic model for Cl^- and HCO_3^- transfer across the gill of *Carassius auratus*. In contact with the external medium, the carrier is characterized by a high affinity for Cl^- and a lower affinity for HCO_3^-. These ions are transported inwards according to the affinities of the carrier for Cl^- and HCO_3^- respectively and the prevailing ionic concentration. In contact with the internal medium (cell interior) conformational changes of the carrier occur characterized by an inversion of the relative affinities for Cl^- and HCO_3^-. These ions are transported outwards according to the new affinities of the carrier. This simplified model implies a one for one exchange between HCO_3^- and Cl^-. It implies the possibility of a Cl^-/Cl^- exchange diffusion mechanism. It explains the reversed Cl^-/HCO_3^- exchange in the presence of external SCN^-, when the affinity of the carrier for Cl^- is lowered.

It is of interest that under SCN^- inhibition the observed absorption of HCO_3^- from the external medium takes place across the gill against both concentration and electrical gradients. The potential ($\phi_{int} - \phi_{ext}$) is $-26{\cdot}7$ mV (see above) and the HCO_3^- concentration in the goldfish plasma is about 10 mM (de Renzis & Maetz, 1973) while that of the external medium is certainly less than titratable alkalinity, i.e. about 2 mM (see Fig. 4). In this case active transport obviously results from a coupled transport, chloride loss down the electrochemical gradient being the driving force for up-hill HCO_3^- uptake.

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