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A Cl^-/HCO_3^--ATPase IN THE GILLS OF *CARASSIUS AURATUS*

ITS INHIBITION BY THIOCYANATE

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Summary

An ATPase is demonstrated in plasma membrane fractions of goldfish gills. This enzyme is stimulated by Cl^- and HCO_3^-, inhibited by SCN^-.

Biochemical characterization shows that HCO_3^- stimulation (K_m = 2.5 mequiv./l) is specifically inhibited in a competitive fashion by SCN^- (K_i = 0.25 mequiv./l). The residual Mg^{2+}-dependent activity is weakly affected by SCN^-.

In the microsomal fraction chloride stimulation of the enzyme occurs in the presence of HCO_3^- (K_m for chloride = 1 mequiv./l); no stimulation is observed in the absence of HCO_3^-. Thiocyanate exhibits a mixed type of inhibition (K_i = 0.06 mequiv./l) towards the Cl^- stimulation of the enzyme.

Bicarbonate-dependent ATPase from the mitochondrial fraction is stimulated by Cl^-, but this enzyme has a relatively weak affinity for this substrate (K_m = 14 mequiv./l).

Introduction

Since the work of Kasbekar and Durbin [1], anion-sensitive ATPase have been demonstrated in a wide variety of tissues which are the site of HCO_3^-, Cl^- or H^+ transport. Previous research has been concerned with gastric mucosa [2,3], pancreatic cells [4], tissues of the salivary ducts [5], kidneys [6] and the liver [2]. A bicarbonate-sensitive ATPase was demonstrated in the gills of *Necturus* [7] and in the gills of trout adapted to fresh water or sea water [8].

Thiocyanate, which is known to inhibit acid secretion by the stomach, has shown itself to be a good inhibitor of chloride transport in the gill. Thus, thiocyanate inhibits the active secretion of Cl^- by eel gills in sea water [9], the active absorption of Cl^- by trout in fresh water [8] and by *Carassius* in fresh

Abbreviation: HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid.

water [9]. The chloride pump of the goldfish gill, characterized by Cl^-/HCO_3^- exchange, is reversed by SCN^- [10]. The study of the effect of SCN^- on the anion ATPase activity in the presence of Mg^{2+} demonstrated that SCN^- strongly inhibits the enzymatic hydrolysis of ATP.

Since an anion-activated, SCN^--inhibited ATPase exists in tissues which actively transport HCO_3^-, Cl^- or H^+, various authors have put forward the hypothesis that this activity furnishes energy for the active transport of these ions. In addition, the enzyme would be regulated by these ions, analogously to the mechanism operating in Na^+/K^+ transport.

Nevertheless, no convincing demonstration as yet exists that this enzyme actually participates in the active transports mentioned. In particular, no clear stimulation by chloride was shown in those epithelia actively transporting Cl^-, such as gills. Also, an SCN^--inhibitable activity which is predominantly localized in mitochondria [11,12] argues against this idea; the enzymatic activities associated with active ion transport would be expected to be found in the microsomal fraction which contains plasma membranes. In addition, it has been shown in *Anguilla rostrata* that the SCN^--sensitive enzymatic activity is not correlated with the activity of the chloride pump when the animal is transferred from fresh to sea water [12].

Although SCN^- inhibition is often noted, the mode of action of this inhibitor has not been precisely delineated. In particular, a specific inhibition of anion stimulation has not yet been clearly demonstrated.

The purpose of the present investigation, then, was to biochemically analyze the anion-dependent ATPase activity in the goldfish gill with emphasis on the effect of Cl^- and HCO_3^- on the enzyme from various subcellular fractions. The effect of SCN^- on the HCO_3^- and Cl^- stimulation was studied with a view towards defining the mode of action of this inhibitor. The results are discussed in reference to knowledge of HCO_3^-/Cl^- exchange and its inhibition by SCN^- in vivo in the goldfish.

Materials and Methods

Preparation of enzyme extracts. Goldfish (*Carassius auratus*), weighing 100 g on the average, were kept un-fed in tanks of running fresh water. The animals were killed and blood was removed from the gills by perfusing with 0.3 M heparinized sucrose solution. Gill filaments were removed and homogenized in 10 ml of the sucrose solution with an Ultraturax mixer. The homogenate was then filtered through nylon gauze and centrifuged at $900 \times g$ for 10 min. A portion of this suspension was retained for determination of enzymatic activities on the crude homogenate and the remainder was centrifuged at $10\,000 \times g$ for 10 min to obtain the mitochondrial fraction (M: $100\,000 \times g \cdot$ min). The resulting pellet was resuspended in the sucrose solution. The supernatant was centrifuged at $20\,000 \times g$ for 35 min and the resulting pellet, containing light mitochondria and heavy microsomes, was resuspended (m: $700\,000 \times g \cdot$ min). The last supernatant was then centrifuged at $110\,000 \times g$ for 45 min to yield the microsomal fraction ($5 \cdot 10^6 \times g \cdot$ min). As most of the experiments were only concerned with the microsomal fraction, one centrifugation at $20\,000 \times g$ for 35 min combined fraction M and m.

Homogenization of tissues was done on ice and the centrifugations were performed at 4°C. Several experiments were done on extracts first treated with Triton X-100 according to Wiebelhaus et al. [7].

Continuous sucrose gradient. Several experiments were performed in order to ascertain the degree of contamination of the microsomal fraction by mitochondria. After resuspension of the pellet $5 \cdot 10^6 \times g \cdot$ min aliquots were placed on top of a linear sucrose gradient (25—60%) and centrifuged at 100 000 $\times g$ for 16 h.

$(Na^+ + K^+)$- and HCO_3^--dependent ATPase activities were continuously measured by an automated technique [13]. The protein content is continuously monitored with the help of a photometer UVICORD (280 nm). Succinate dehydrogenase activity and protein concentrations were measured in successive samples after fractionating the gradient with a collector.

Enzyme assays. ATPase activity was determined with an automated method [13].

Anion-dependent activity was determined in the following reaction medium: 50 mM HEPES/Tris, pH 8.2, 10^{-4} M ouabain, 0.5 mM ATP-Mg^{2+}. In certain experiments the medium was buffered with bicarbonate. The particular condition of pH and ion concentration are described below for each experiment.

The $(Na^+ + K^+)$-dependent activity was measured as the difference in enzymatic activity in the absence or presence of 10^{-4} M ouabain in a medium buffered with 50 mM HEPES/Tris, pH 7.2, containing 60 mM NaCl, 20 mM KCl and 0.5 mM ATP-Mg^{2+}.

The concentration of protein in the reaction mixtures was on the order of 5—15 μg/ml for the homogenate, 2—6 μg/ml for heavy mitochondria and 5—10 μg/ml for microsomes.

Enzymatic hydrolysis was performed for 10 min at 27°C and the reaction was stopped by 10% trichloroacetic acid/3.5% sodium dodecyl sulfate. Inorganic phosphate was determined colorimetrically in the presence of ammonium molybdate and ascorbic acid [14].

Succinate dehydrogenase activity was measured utilizing the method of Clark and Porteous [15], using iodonitrotetrazolium violet as electron acceptor.

Protein concentration was determined with the method of Lowry et al. [16].

Results

Bicarbonate dependence

Characteristics of HCO_3^- stimulation. The ATPase activity of various subcellular fractions was studied as a function of the sodium bicarbonate concentration in the reaction medium. The results clearly show a bicarbonate-dependent activity with a maximum at a bicarbonate concentration of 18—20 mequiv./. Bicarbonate stimulation of the microsomal enzyme is shown in Fig. 1a: the activity measured for each HCO_3^- concentration minus the activity measured in the absence of HCO_3^- is expressed as percent of maximum stimulation obtained.

The affinity constant of the enzyme of HCO_3^-, determined from the methods of Lineweaver-Burk and Eadie-Hofstee, is 2.5 mequiv./l. (Fig. 1b). Values of this

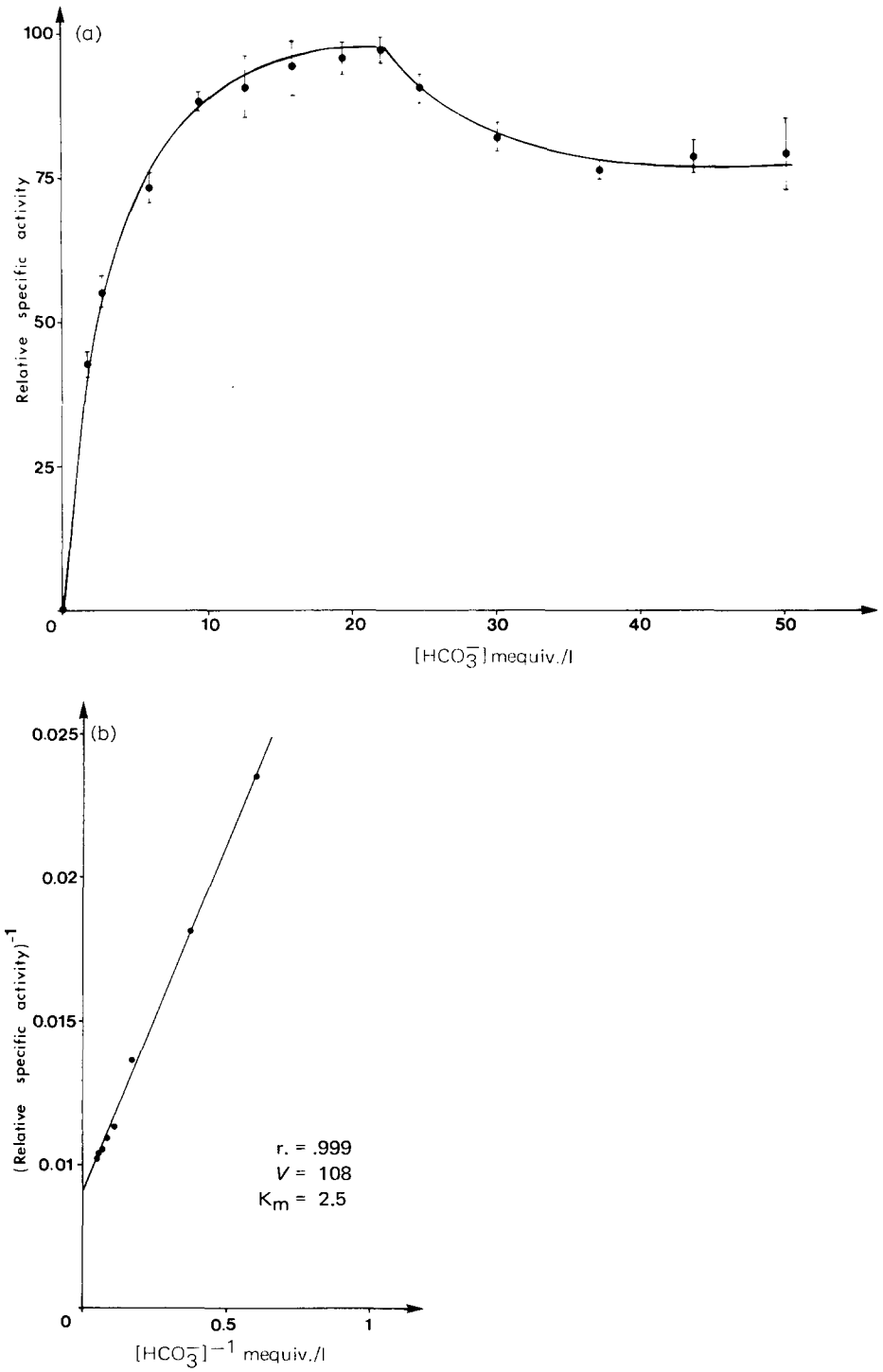

Fig. 1. (a) Variation of bicarbonate-dependent ATPase activity as a function of bicarbonate concentration. The results shown were obtained with the microsomal fraction. The average of four experiments with different extracts are shown (±S.E.). (b) Lineweaver-Burk plot of the initial eight points of a, used to determine K_m.

constant for the enzyme in the homogenate and the mitochondrial fraction are practically identical: for the homogenate K_m = 2.5 mequiv./l and for the mitochondrial fraction (M) K_m = 4.1 mequiv./l.

It should be noted that in reactions carried out in the absence or presence of 50 mM HCO_3^-, the variations in pH of the media do not exceed 0.15 unit.

Distribution of HCO_3^--dependent ATPase. The specific activities of the HCO_3^--ATPase of different subcellular fractions, determined in the presence of 18 mequiv./l HCO_3^-, are shown in Table I. Residual ATPase activity was measured in the presence of 10^{-2} M SCN^- or in the absence of bicarbonate. The bicarbonate-sensitive activity was thus calculated by two independent methods.

The results demonstrate that all the fractions studied contain an important HCO_3^--sensitive ATPase activity. The fraction containing heavy mitochondria posesses the highest measured specific activity and the greatest difference was noted between this fractions and that of microsomes. The difference is even greater, with the residual activity of these two fractions measured in the presence of SCN^- or in absence of HCO_3^-. In effect residual activity in the mitochondrial fraction (100 000 $\times g \cdot$ min) was quite different from that measured in the absence of HCO_3^-, whereas the two residual activities in the microsomal fraction were comparable.

In Table I are shown the activities of $(Na^+ + K^+)$-dependent ATPase as well as of succinate dehydrogenase. The relatively low succinate dehydrogenase activity of the microsomal fraction argues for minimal contamination by mitochondria. Also, there is a very slight $(Na^+ + K^+)$-dependent ATPase activity in mitochondria (M) and a slight residual activity in the microsome fraction. These

TABLE I

ANION-DEPENDENT ATPase, $(Na^+ + K^+)$-ATPase AND SUCCINATE DEHYDROGENASE ACTIVITY IN THE GOLDFISH GILLS

Activities in μmol P_i/h per mg protein or nmol formazan/h per mg protein.

	Homogenate	Mitochondria		Microsomes
		Heavy M: 100 000 $\times g \cdot$ min	Light m: 700 000 $\times g \cdot$ min	
HCO_3^--ATPase				
HCO_3^- = 0 vs. HCO_3^- = 18 mM	4.3 ± 0.25	15.1 ± 3.99	5.9 ± 1.80	5.6 ± 1.85
HCO_3^- vs. SCN^-	6.8 ± 0.33	27.7 ± 5.38	7.8 ± 2.25	6.1 ± 2.09
Residual activity				
HCO_3^- = 0 mM	8.5 ± 0.04	25.0 ± 3.29	18.8 ± 4.95	11.5 ± 3.35
SCN^-	6.0 ± 0.12	12.4 ± 1.88	16.9 ± 4.38	10.9 ± 3.03
Cl^- activation *	3.4 ± 0.29 (n = 7)	10.8 ± 2.09	3.0 ± 0.88	2.4 ± 0.41 (n = 7)
$(Na^+ + K^+)$-ATPase **	0.6 ± 0.10	1.8 ± 0.31	7.9 ± 1.66	4.9 ± 0.41
Residual activity **	8.5 ± 0.34	40.2 ± 1.81	44.3 ± 6.54	12.6 ± 0.97
Succinate dehydrogenase activity	100.2 ± 9.63 (n = 10)	229.7 ± 44.1 (n = 4)	232.7 ± 54.1 (n = 4)	18.3 ± 4.79 (n = 17)

* Activities measured in the presence of bicarbonate and chloride.

** Activities measured after solubilization by deoxycholate.

facts demonstrate the existence of a HCO_3^--dependent ATPase activity which is specific to the microsomal fraction.

Confirmatory evidence was obtained by the investigations making use of sucrose gradients in an effort to further purify this microsomal fraction.

These results were found reproducible in three separate runs. In Fig. 2 ($Na^+ + K^+$)- and HCO_3^--dependent ATPase activities are plotted against sucrose concentrations. It may be seen that an excellent correlation prevails between these activities. The parallel distribution of the two enzymes clearly suggests that the bicarbonate-ATPase is present in the plasma membranes. Within the limits of the sensitivity of our technique no succinate dehydrogenase activity could be detected in the various fractions. Thus, contamination by mitochondria in the microsomal fraction studied above must be negligible.

In view of these observations, purification of the microsomal fraction was not pursued and the kinetic studies reported in the forthcoming paragraphs were performed on the $5 \cdot 10^6 \times g \cdot$ min fraction.

Effect of thiocyanate on bicarbonate stimulation. The effect of various concentrations of SCN^- on the microsomal fraction was examined in the presence of two concentrations of HCO_3^-, 10 and 20 mequiv./l. Bicarbonate activation was measured as the difference in P_i liberated in the presence and absence of the two concentrations. It can be seen (Fig. 3) that SCN^- competitively inhibits ATP hydrolysis by the HCO_3^--dependent ATPase. K_i (the abscissa of the intersection point of the two lines) equals 250 μequiv./l.

The inhibitory effect of SCN^- on activity was also studied on the homogenate in the absence or in the presence of 25 mequiv./l of HCO_3^-. The results, obtained from four different animals (Fig. 4a) clearly show that the ATPase activity measured in the absence of HCO_3^- is only slightly inhibited by SCN^-. Bicarbonate-dependent activity tends towards zero for SCN^- concentrations

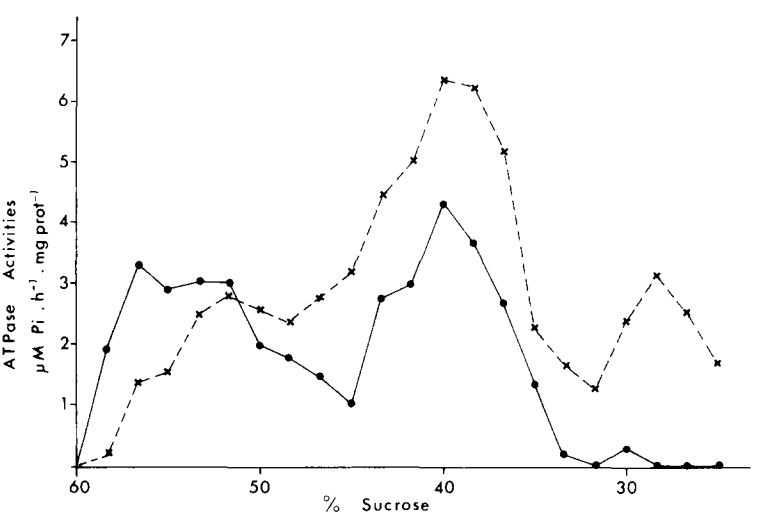

Fig. 2. Distribution of HCO_3^--ATPase (•) and ($Na^+ + K^+$)-ATPase (×) in the continuous sucrose gradient. 0.5 ml of a mixture of the microsomal fractions from six different animals was placed on top of a 25—60% linear sucrose gradient. Anion-ATPase activity was measured in the presence of 20 mequiv./l HCO_3^-. When the HCO_3^--ATPase activity is plotted against the ($Na^+ + K^+$)-ATPase activity a good correlation is obtained: $y = 0.47\ (\pm 0.039)x + 0.44\ (\pm 0.306)$, $n = 23$, $r = 0.577$, $P < 0.01$.

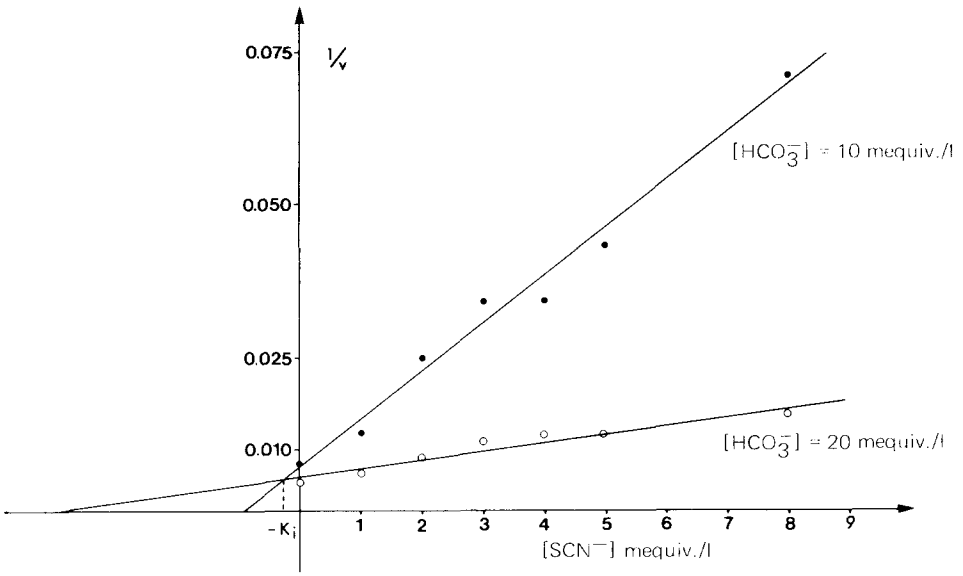

Fig. 3. Dixon plot used to determine the type of inhibition by SCN^- of bicarbonate-dependent activity. The results are the average of three determinations on the microsomal fraction. The reciprocal of the difference between P_i liberated in the absence and in the presence of two concentration of HCO_3^- (10 and 20 mequiv./l) is plotted as a function of SCN^- concentration in the reaction mixture.

greater than 10^{-2} M: at this concentration of inhibitor the activity measured in the presence of 25 mequiv./l HCO_3^- is practically the same as that measured in the absence of both SCN^- and HCO_3^-.

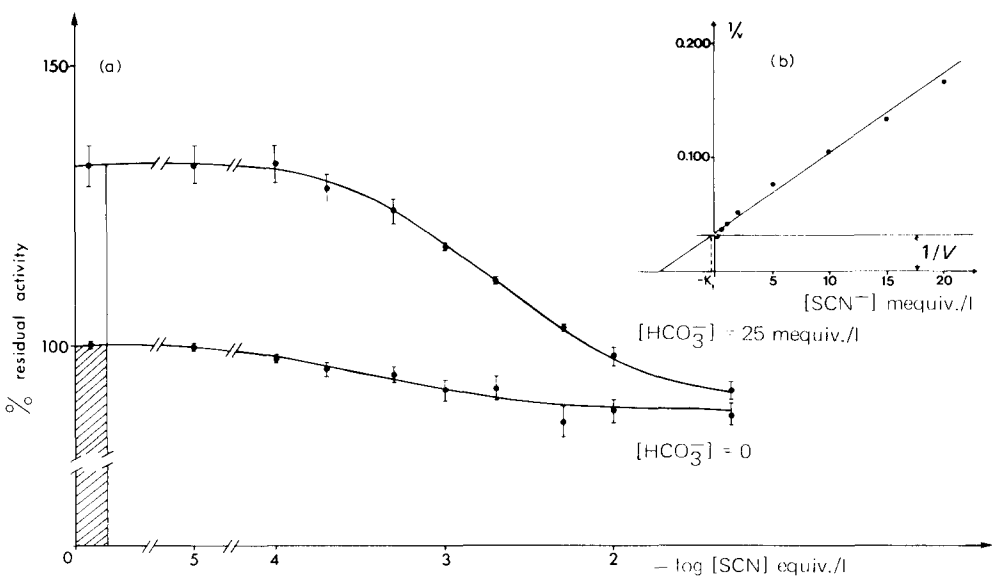

Fig. 4. (a) Variation of bicarbonate-dependent activity and residual activity as a function of SCN^- concentration in the reaction medium. The results were obtained with four different gill preparations from four different animals. Results are expressed as percent of activity measured in the absence of bicarbonate and thiocyanate. (b) Dixon plot used to determine K_i. The reciprocal of the percent of activity due to HCO_3^- is expressed as a function of SCN^- concentration.

An analysis was performed, according to the method of Dixon, to determine the affinity constant of the enzyme for SCN^- (K_i). The reciprocal of percentage of activation by HCO_3^-, for different SCN^- concentrations, is shown in Fig. 4b. For each SCN^- concentration, bicarbonate-dependent activity was determined as the difference in activities measured in the presence and in the absence of HCO_3^-. These values are expressed as percent of the residual activity measured in the absence of SCN^- and HCO_3^-. The intercept of the ordinate ($1/V = 0.034$) shows that in this experiment the bicarbonate-dependent activity represents about 33% of the residual activity.

As shown above, SCN^- is a competitive inhibitor of HCO_3^-. The representation chosen easily permits the calculation of K_i. The curve obtained intersects the baseline at the point $i = -K_i\ (S/K_m + 1)$ where S is the substrate concentration and K_m is the affinity constant of the enzyme for HCO_3^-. In addition, the value of K_i can also be obtained by tracing a line parallel to the abscissa and passing through the point $1/V$. In this case, the point at which the two lines intersect will have $-K_i$ as abscissa. The two methods utilized yield a value of K_i, the affinity constant of the enzyme for SCN^-, equal to 400 μequiv./l.

Thus, these results clearly show that SCN^- is a competitive inhibitor of bicarbonate activation. This inhibitory effect is consistent with the fact that a concentration of 10^{-2} M SCN^- lead to a total inhibition of HCO_3^- stimulation and that, in the microsomal fraction, the activities of the ATPase measured in the absence of HCO_3^- or in the presence of SCN^- are identical (Table I).

Effect of pH. The effect of pH on enzymatic hydrolysis of ATP by the microsomal fraction are shown in Fig. 5. Residual ATPase was measured in the presence of SCN^-. The pH gradient was obtained by varying the ratio HEPES/Tris. It is found that bicarbonate-stimulated activity is far more sensitive to variations in pH than is residual activity. The pH optimum is between pH 8.0 and 8.25.

Comparable experiments on the homogenate and the mitochondrial fractions

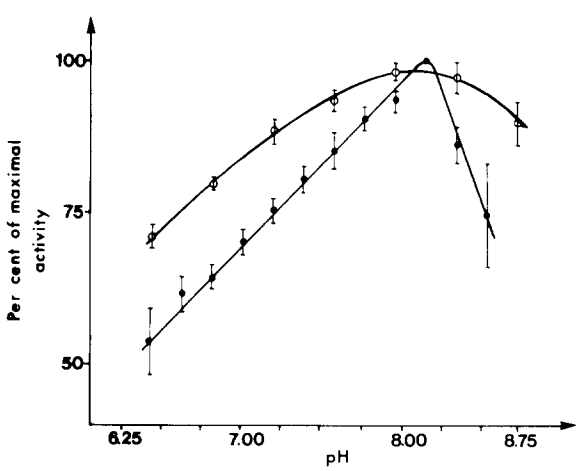

Fig. 5. Effect of pH on bicarbonate-dependent (full circles) and residual (open circles) ATPase activities expressed as percent of the maximal activities measured. The experiments were done on microsomal extract from four different animals.

(M + m) indicate no differences among the various fractions, in activity or pH optimum.

Effect of other cations. In Table II are shown the activities of the ATPase stimulated by 18 mequiv./l HCO_3^- in the presence of various cations. Replacement of Na^+ by K^+ or NH_4^+ leads to no significant differences in activity. The only exception seems to be Tris, which exhibits an important inhibition at elevated concentrations (100 mM).

On the other hand when Mg^{2+} is replaced by Ca^{2+}, two different effects are noted. First an increase in the residual activity measured in the presence of SCN^- can be observed, which characterizes a Ca^{2+}-dependent ATPase previously described in the trout by Ma et al. [17]. Secondly, there is a large reduction in bicarbonate stimulation, demonstrating the necessity of Mg^{2+} in order to obtain this effect. Analogous observations were obtained for pancreatic ATPase [18] and for the anion-sensitive ATPase of the midgut of the larvae of *Hyalophora cecropia* [19].

Chloride dependence

The effect of increasing concentrations of Cl^- on the enzymatic hydrolysis of ATP was studied, with particular attention to the ATPase from the mitochondrial and microsomal fractions.

Microsomal fraction. In the absence of HCO_3^-, no stimulation could be obtained with Cl^- (Fig. 6). To eliminate the possibility of interference with Tris at high concentrations (>30 mM), the reactions were buffered with 5 mM HEPES/Tris, pH 8.0.

The concentration of plasma bicarbonate in the goldfish is 9 mequiv./l [20]. Since intracellular pH is known to be more acidic than plasma pH, it seemed reasonable to study the possible effects of chloride in the presence of 5 mequiv./l HCO_3^- and in the absence of HEPES/Tris. Measurements of pH (8.0) during the reaction showed fluctuations not exceeding 0.1 unit. Under these conditions of incubation, in the range of Cl^- concentrations of 0—15 mequiv./l, a stimulation of ATPase activity was noted which followed Michaelis-Menten kinetics (Fig. 7).

The maximum stimulation observed was at 12 mequiv./l and represents about 60% of the activation observed with 5 mequiv./l of HCO_3^- alone (Fig. 8). This is

TABLE II

EFFECT OF DIFFERENT CATIONS ON THE ATPase ACTIVITY OF THE MICROSOMAL FRACTION MEASURED IN THE PRESENCE OF HCO_3^- (18 mequiv./l) AND WITH OR WITHOUT SCN^- (10 mequiv./l)

Results are expressed as percent of residual activity measured in the presence of Na^+ and Mg^{2+}.

	Na^+ (n = 8)	K^+ (n = 8)	NH_4^+ (n = 4)	Mg^{2+} (n = 4)	Ca^{2+} (n = 4)
Residual activity (+SCN^-)	100	101.0 ± 0.50	102.5 ± 0.69	100	117.8 ± 2.70 *
Bicarbonate-dependent activity	34.9 ± 8.50	34.0 ± 8.10	27.6 ± 7.81	25.8 ± 5.44	4.1 ± 1.66 *

* $P < 0.1$.

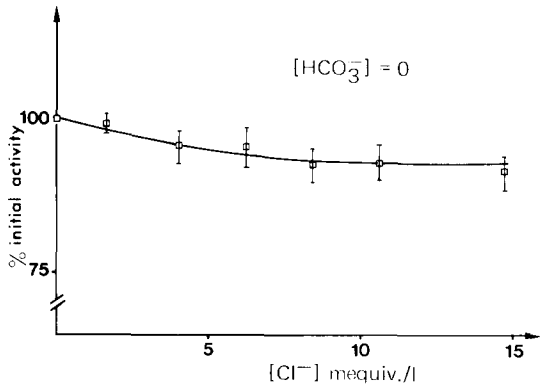

Fig. 6. Effect of different Cl^- concentration on ATPase activity in the absence of external HCO_3^-. The experiments were done on microsomal extract from four different animals.

independent of the nature of chloride co-ions (Na^+, K^+, NH_4^+, choline).

The K_m of the enzyme for Cl^- was determined with the method of Lineweaver-Burk and Eadie-Hofstee and is equal to 1.15 mequiv./l.

The microsomal fraction was examined in the presence of 18 mequiv./l HCO_3^- (pH adjusted to 8.0 with HEPES). The Cl^- effect under these conditions leads to an additional stimulation of activity which is 41 ± 7% that of bicarbonate ($P < 0.01$: $n = 4$).

Inhibition of enzyme activity is noted with Cl^- concentrations exceeding 20 mequiv./l. A concentration of 95 mequiv./l Cl^- leads to 60% of the inhibition provoked by 10 mequiv./l SCN^-.

Mitochondrial fraction. In the presence of 5 mequiv./l bicarbonate, the effect of Cl^- on the HCO_3^--ATPase of the 100 000 × g · min fraction (M) is stimulatory. The K_m of the enzyme for Cl^-, determined by the Lineweaver-Burk method, is 14 mequiv./l (Fig. 8).

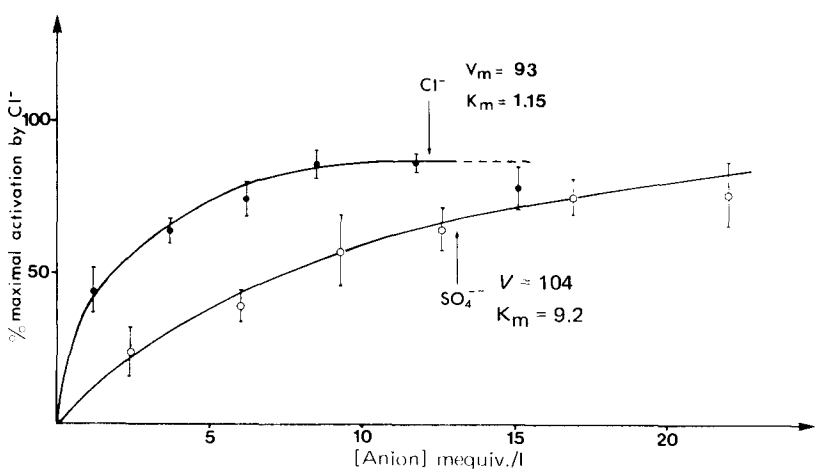

Fig. 7. Effects of different concentrations of chloride (full circles) and sulfate (open circles) on the anion-dependent ATPase activity of the microsomal fraction. Experiments were done in the presence of 5 mequiv./l HCO_3^- in the reaction mixture. Results are expressed as percent of maximum activation by Cl^- corresponding to 11 determinations for Cl^- and four for SO_4^{2-}.

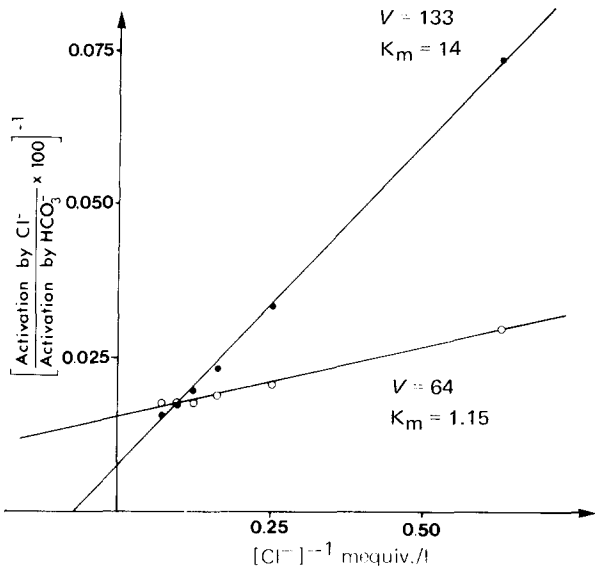

Fig. 8. Lineweaver-Burk plot showing activation by different Cl^- concentrations, expressed as percent activation by 5 mequiv./l HCO_3^-. Full circles: M fraction, curve constructed from five determinations. Open circles: microsomal fraction, curve constructed from 10 determinations.

In Table I are shown the activities of the chloride-dependent enzyme measured in the presence 5 mequiv./l of HCO_3^- and 12 mequiv./l of Cl^-.

Effect of SCN^- on Cl^- stimulation. Chloride-stimulated ATPase activities were determined in the presence of various concentrations of chloride and in the absence or the presence of two concentrations of SCN^- which have slight effects on HCO_3^- stimulation: $5 \cdot 10^{-5}$ M and $5 \cdot 10^{-4}$ M. Experiments were performed using the microsomal fraction in the presence of 5 mequiv./l of HCO_3^-. Chloride activation was estimated as the difference in activities in the presence of a given concentration of Cl^- and zero Cl^-. The data were analyzed to obtain the affinity constant for Cl^- in the absence of SNC^- (K_m) and in its presence (K_x). The affinity constant for SCN^- (K_i) was also determined, as were the values of maximum activation when the inhibitor was present (V_x).

For each concentration of chloride, in the presence or absence of SCN^-, Cl^- activation was caculated as the percent of maximum activation observed in the absence of SCN^- (Fig. 9).

The affinity constant of the enzyme for Cl^- in the absence of SCN^- is, in this particular experiment, 0.750 mequiv./l. The two curves obtained in the presence of SCN^- show that Cl^- activation is inhibited and that the inhibition is of a mixed type. The presence of the inhibitor leads to a simultaneous variation in the K_m for Cl^- and of the maximum activation.

The results were analyzed according to the methods recommended by Dowd and Riggs [21], where V is represented as a function of V/S and S/V is represented as a function of S. This type of calculation confirms the values shown on Fig. 9. The affinity constant of the enzyme for inhibitor was calculated using formulas given by Dixon and Webb [22]:

$$\frac{1}{K_x} = \frac{1 + iK_m/K_iK'_m}{K_m(1 + i/K_i)} \quad \text{and} \quad \frac{1}{V_x} = \frac{1 + iK_m/K_iK'_m}{V}$$

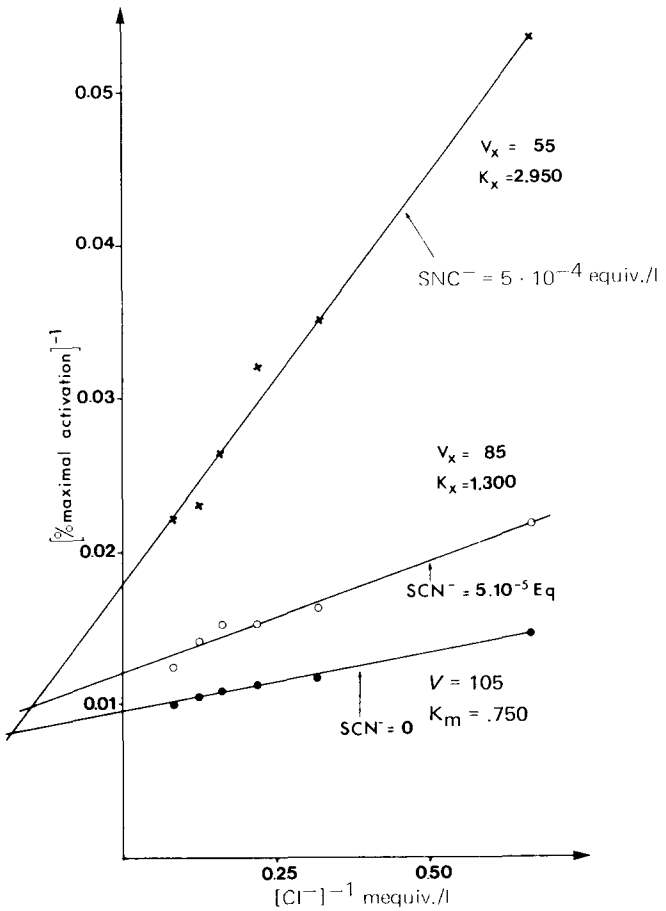

Fig. 9. Reciprocal of the percent of maximum activation by Cl^- as a function of the reciprocal of Cl^- concentration. Maximum activation by Cl^-, was measured in the presence of 12.5 mequiv./l Cl^- and in the absence of SCN^-. The experiments were done either in the absence of SCN^- (full circles) or in the presence of two different concentrations of SCN^-: open circles, $5 \cdot 10^{-5}$ M; crosses, $5 \cdot 10^{-4}$ M. Each point is the average of three determinations on a mixture of the microsomal fractions from four different animals.

Combining these two equations gives:

$$\frac{K_x}{V_x} = \frac{K_m(1 + i/K_i)}{V}$$

By replacing K_x, V_x, K_m and V by their numerical values, the value of the affinity constant for SCN^- is found to be 60 μequiv./l.

It should be noted that with the SCN^- concentrations mentioned above, no inhibition of chloride activation occurs for the ATPase of the mitochondrial fraction (M).

Effects of other anions. The ions studied until now are those which intervene directly in gill transport and have a direct effect on the microsomal ATPase, either inhibitory (SCN^-) or stimulatory (HCO_3^-, Cl^-). Nevertheless, other ions act on this ATPase activity, including sulfate and acetate.

In the presence of 5 mequiv./l HCO_3^-, ATPase activity of the microsomal frac-

tion is stimulated by sulfate. The results are shown in Fig. 7, from which it can be calculated that the K_m for SO_4^{2-} is 8 mequiv./l. As for chloride no stimulation by sulfate is observed in the absence of HCO_3^-. In the presence of 18 mequiv./l HCO_3^-, the stimulation by sulfate occurs again between 0 and 25 mequiv./l. Concentrations greater than 25 mequiv./l provoke an inhibition of the enzyme and at 100 mequiv./l SO_4^{2-}, the inhibition of 20% of that obtained with 10 mequiv./l SCN^-.

At the last mentioned HCO_3^- concentration, acetate ion is only an activator, with a maximum around 30 mequiv.

As has already been observed [3,6] nitrate is inhibitory, its effect being proportional to its concentration.

HEPES, between 0 and 50 mM, has no effect on ATPase activity measured in the presence of HCO_3^-.

The possibility remains that stimulation by sulfate or chloride could reflect an osmolality effect on enzyme activity. This seems unlikely for three reasons. First, in the absence of bicarbonate, these ions are unable to activate the ATPase. Second HEPES has no effect between 0 and 50 mM. Third, osmolality effects on $(Na^+ + K^+)$-ATPase investigated in the outer renal medulla by Alexander and Lee [23] indicate that this type of activation becomes significant only above 300 mosM. In the present study stimulatory effects of Cl^- and SO_4^{2-} were observed for osmolalities lower than 100 mosM.

All the anions studied, except HEPES, were added as their sodium salts, thus any inhibitory effects observed (Cl^-, SO_4^{2-}, NO_3^-) would not be due to the accompanying cation. Nevertheless, an inhibitory effect of certain cations, such as Tris, especially at high concentrations, is not excluded.

Effect of Triton X-100. When the microsomal fraction is solubilized with this detergent, bicarbonate activation increases 2.5-fold. In the presence of 5 mequiv./l HCO_3^-, the characteristics of Cl^- stimulation are unchanged.

Discussion

Although transmembrane ion transport has been the subject of intensive research and has been copiously discussed, the mechanisms underlying this phenomenon, remain to be elucidated. The discovery and study of an anion-sensitive, thiocyanate-inhibited ATPase lends credence to the idea that this enzyme would furnish energy for the active transport of anions, in the same manner that the mechanism implicated in Na^+-K^+ exchange.

The present research was undertaken to study this hypothesis with a biological system in which anion transport (Cl^-, HCO_3^-, SO_4^{2-}, SCN^-) has been well studied: the goldfish gill.

Many authors have reported that the gills of goldfish and trout are the site of active transport of Cl^- in fresh water against an electrochemical gradient [10,20,24–26].

Chloride absorption in the goldfish is bound to the bicarbonate excretion. In the presence of SCN^- in the external medium, the exchange is reversed, suggesting that this exchange is obligatory. Since the gills are not permeable to SCN^-, this observation suggest that the mechanism responsible for Cl^- transport is located on the apical surface. The influx of Cl^- varies as a function of its

concentration in the external medium and obeys Michaelis-Menten kinetics with a K_m of 100 μequiv./l. Thiocynate reversibly inhibits this flux with an inhibition of a mixed type (K_i = 10 μequiv./l). This inhibition involves neither an effect on carbonic anhydrase nor is a result of absorption of SCN^- in the place of Cl^-. Studies with sulfate showed that this ion is only slightly transported by the goldfish gill [24,27].

The present results show that the epithelium of this organ contains an ATPase which is activated by HCO_3^- and Cl^- and is inhibited by SCN^-. The principal characteristics of this enzyme are the following: (1) activity is found in all subcellular fractions, especially in plasma membranes in the absence of contamination by mitochondria; (2) bicarbonate stimulation is demonstrable only in the presence of Mg^{2+} (see Table II); (3) bicarbonate stimulation is specifically inhibited competitively by SCN^- (see Figs. 3 and 4); (4) in the microsomal fraction, this activity is stimulated by Cl^- (Fig. 7) in the presence of HCO_3^- (K_m = 1 millequivalent per litre); (5) thiocyanate inhibits chloride stimulation, the inhibition being mixed, specific, and not due to an inhibition of HCO_3^- stimulation, for low concentrations of SCN^-. The affinity of SCN^- for HCO_3^- site (K_i = 250 μequiv./l) is indeed much less than that for the Cl^- site (K_i = 60 μequiv./l).

The intervention of an ATPase in Cl^- transport, postulated by numerous authors, was recently put in doubt by Solomon et al. [12]. These authors were interested in the Mg^{2+}-dependent activity inhibited by SCN^- and the predominantly mitochondrial localization of this activity in the gill of *Anguilla rostrata* did not allow them to correlate this activity with the anion transport. In Table I, the results dealing with SCN^- inhibition confirm these observations. For the mitochondrial fraction (M), the values of the residual ATPase activity are different depending on their measurement in the presence of SCN^- or in the absence of HCO_3^-. This indicates that in this fraction SCN^- inhibits not only the HCO_3^--dependent ATPase, but a part of the Mg^{2+}-dependent enzyme as well. Comparable observations were made on the mitochondrial fraction of rat liver, [28], where it was observed that in the absence of HCO_3^- in the incubation medium, 10^{-2} M SCN^- produced a 55—65% inhibition of ATPase activity. It has been observed in the submandibular gland of the rabbit, as in the present report, that thiocyanate has a different action on residual ATPase of mitochondria and microsomes. Mitochondrial activity is inhibited 42% whereas microsomal activity is inhibited only 13% by $2 \cdot 10^{-2}$ M SCN^- [5].

It was shown by Soumarmon et al. [11] that in rat gastric mucosa, SCN^- provokes strong inhibition of mitochondrial ATPase; the microsomal enzyme is only slightly inhibited under the same conditions. These observations explain the weak activity observed by Solomon et al. [12] in the microsomal fraction; their results are not an argument against the participation of an SCN^--sensitive ATPase in anion transport. In fact, the results of Kerstetter and Kirschner [8], as well as the above-mentioned results, clearly show that there exists in the gills of fish and HCO_3^--dependent, SCN^--inhibited ATPase. The specific inhibition of this activity by SCN^- (Fig. 4) shows that it is different from a Mg^{2+}-dependent ATPase which is modulated by anions.

Certain results in the present report are apparently inconsistent with a strict correlation between characteristics of fluxes and those of the ATPase.

Although the relationship between K_m and K_i are similar in vivo and in vitro, the values of the affinity constants for Cl^- and SCN^- are different. The characteristics of ionic transport measured in vivo are the resultants of physiological mechanisms which account for the complexity of gill epithelium structure and the ionic microenvironment in which the transporting cells are found. Histochemical studies by Bierther [29], for example, show that Cl^- are concentrated by the cell coat in the apical region of the cells. It is thus difficult to directly compare the accessibility of ions to transporter sites and the probability of fixation of ions on an enzyme molecule in vitro.

The results also show that sulfate, which is hardly transported, stimulates the HCO_3^--ATPase. It should be noted that, on the one hand, the affinity of the enzyme for SO_4^{2-} is quite smaller than that for Cl^- and, on the other hand, the accessibility of this ion to the transporter is probably more difficult, given the size and charge differences between it and Cl^-

These objections do not seem sufficient to warrant a rejection of the hypothesis that an anion-sensitive ATPase, such as the one described in the present report, is implicated in Cl^-/HCO_3^- exchange in the gills.

A bicarbonate-dependent, chloride-activated ATPase in the plasma membranes, as well as the specific SCN^- inhibition of these HCO_3^- and Cl^- effects, are consistent with the characteristics of Cl^-/HCO_3^- exchange and its inhibition by thiocyanate.

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