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Role of chloride ions in glycine transport in a sea fish, the bass (*Dicentrarchus labrax*)

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The transport of glycine by vesicles of the bass intestine brush border is dependent on Na^+ and also on the presence of Cl^- in the incubation medium. The optimum pH for the chloride effect is between 5.5 and 6. Other anions are also capable of stimulating the transport of this amino acid, in the following order of decreasing efficacy: $Br^- > Cl^- > I^- > NO_3^- > SCN^- > F^- > S_2O_3^{2-} > SO_4^{2-} > CH_3CO_2^-$. Cl^- mainly acts on one of the two components of glycine transport, the high affinity component, where its principal effect is to increase V_{max} and, to a lesser extent, reduce K_m. The action of Na^+ on the transport of glycine by the high affinity mechanism may be expressed by a Michaelis-Menten equation, the K_m of which is not affected by Cl^- ($\simeq$ 300 mM). The effect of Cl^- on this transport is also fitted by a Michaelis-Menten equation, though its K_m is approximately 10-times lower than that of Na^+. Finally, we showed that the presence of Cl^- has the same effect on the glycine transport kinetics as an increase in the Na^+ concentration and we suggest that Cl^- acts by increasing the accessibility of specific sites on the carrier to Na^+.

Introduction

The transport of amino acids by vesicles of the intestinal brush border of the sea fishes (*Boops salpa* and *Dicentrarchus labrax*), depends on the Na^+ gradient. Such transport is also stimulated by the membrane potential brought about by an outward diffusion of K^+ ions [1–3].

The most original characteristic of these transport mechanisms is their sensitivity to external anions such as Cl^-. Although this dependence varies in intensity in different fish species and for different amino acids, it seems to be a general characteristic.

Previous investigations of the transport of glycine and alpha-aminoisobutyrate demonstrated that the chloride effect:

(a) only concerns the transport of amino acids and not that of sugars; (b) requires the presence of Na^+ outside the vesicles; (c) depends on the pH of the incubation medium; (d) is slightly amplified by a concentration gradient [3].

The aim of this study was to determine the action mechanism of Cl^- and in particular, its interactions with Na^+. This study was carried out using a cultured sea fish, the bass (*Dicentrarchus labrax*).

We determined the ionic specificity of the Cl^- effect and its relation to the pH and Na^+ concentration, by investigating, in particular, the mode of action of Cl^- when the sodium concentration was varied.

Abbreviations: Mes, 4-morpholineethanesulfonic acid; Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic aicd; Tris, tris(hydroxymethyl)aminomethane.

Material and Methods

The experiments were carried out on the bass (*Dicentrarchus labrax*) a teleostean fish from the Mediterranean. The fish used for these studies weighed between 200 and 300 g and came from an CNEXO experimental fish farm.

The technique used to obtain the brush border vesicles has been described previously. The calcium precipitation method was employed which was developed in mammals [4], adapted for the fishes [1] and in particular, for the bass [3].

Vesicles were incubated at 25°C, for 15 s, in media containing 100 μl of buffered saline solution (composition described in the text), and [U-^{14}C]glycine. The reaction was started by adding 20 μl of vesicles that had previously been loaded by incubation for 2 h at 4°C in a buffered saline medium (composition given below). It was stopped by adding 1 ml of a solution with the same composition as the incubation medium that had been cooled on ice. The content of each tube was filtered on a Millipore filter (0.22 μm). The quantities of glycine retained by the filter were then expressed in terms of the weight of protein, determined by the method of Lowry et al. [5], in the vesicle sample.

Results

1. pH dependence

Previous work has shown that the action of Cl^- on amino acid transport is stronger at pH 6.5 than at pH 7.4 [3]. We determined the optimum pH more precisely by testing every half pH unit from 5.5 to 8.

The buffers chosen were: Mes (p*K* 6.1) pH from 5.5 to 6.5, Bes (p*K* 7.1) pH 7 and 7.5, Hepes (p*K* 7.5) pH 8. They were utilized at a concentration of 10 mM and adjusted to the correct pH with Tris base.

During the preparation of the vesicles, these were first taken up in distilled water and then distributed into as many tubes as there were pH values. An appropriate quantity of buffer and K_2SO_4 was then added to each tube, the contents of which were mixed for 10 min. These tubes were then placed at +4°C for at least 2 h before the start of the experiments.

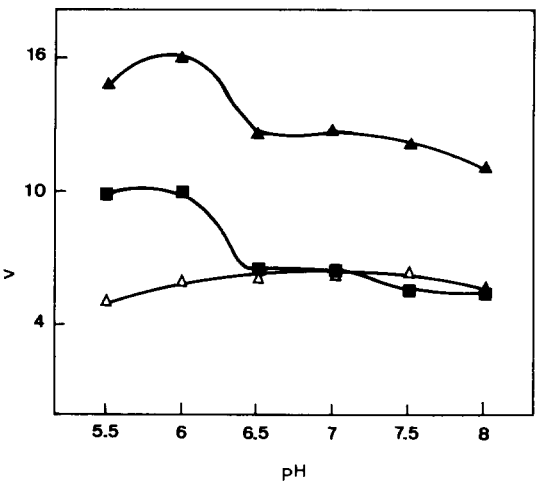


Fig. 1. pH dependence of glycine transport (30 μM). Buffers used: Mes (10 mM) between 5.5 and 6; Bes (10 mM) between 7 and 7.5; Hepes (10 mM) at 8. The incubation medium contains NaCl (100 mM, ▲) or Na_2SO_4 (50 mM, △). The medium inside the vesicles contains K_2SO_4 (50 mM). ■, represents the difference between NaCl and Na_2SO_4. *v* is expressed in μmol/mg protein per 15 s.

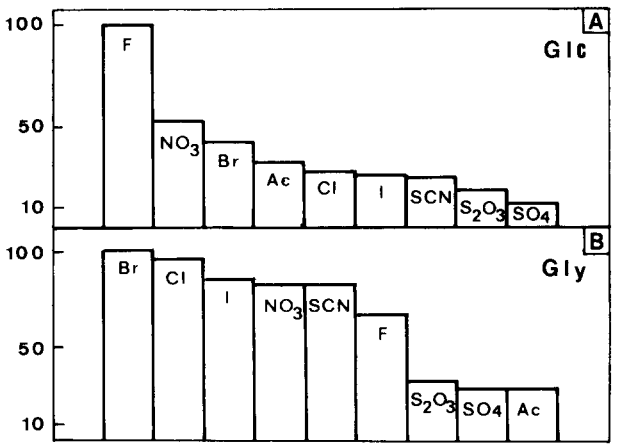
The incubations were performed in NaCl (100 mM) or Na_2SO_4 (50 mM) solution for each pH. Mannitol was used to maintain the same osmotic pressure in these different media. Identical results can be obtained when using solutions of identical tonicity. This has been confirmed for glucose transport using NaCl (100 mM) or sodium methylsulfonate.

The results (Fig. 1) show that the maximum glycine transport occurs at a pH of 6 when the incubation medium contains NaCl. In contrast, in the presence of Na_2SO_4 the pH only has a moderate effect; the maximum is not very pronounced and is clearly shifted towards the more alkaline pH values (7 to 7.5).

The difference between the transport rates with NaCl and Na_2SO_4 gives a more accurate evaluation of the pH at which the Cl^- effect is optimum: at a pH of approx. 5.5. All the subsequent experiments were carried out at this pH.

2. Specificity of the anion .

We compared the efficacy with which certain anions stimulate glycine transport, in the presence of the same external Na^+ concentration (100 mM) and the same K_2SO_4 concentration (50 mM) inside the vesicles.



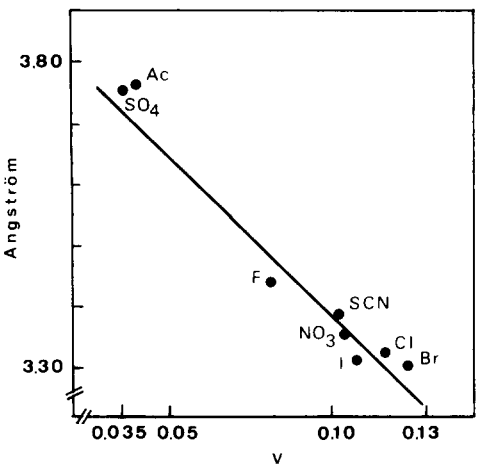


Fig. 2. Effects of anions on glycine (30 μM) and glucose (10 μM) transport at pH 5.5 (Mes: 10 mM). The Na^+ concentration of the incubation medium is 100 mM. The medium inside the vesicles contains K_2SO_4 (50 mM). The values for diffusion have been subtracted. The results are expressed in relation to the highest value.

Fig. 3. Stimulation of the glycine transport by sodium salts: effect of the anion size. The Na^+ concentration is 100 mM. Vesicles are preloaded with K_2SO_4 (50 mM). v is expressed in μmol/mg protein per 15 s.

The results (Fig. 2) show that other anions had an effect similar to Cl^-. Cl^- may be replaced by Br^- which has the same efficacy, and, to a lesser extent, by I^-, NO_3^-, SCN^- and F^-. Other anions, such as SO_4^{2-}, $S_2O_3^{2-}$ or $CH_3CO_2^-$ are much less able to take the place of Cl^- and may therefore be considered to be neutral.

We therefore have, in order of decreasing efficacy:

$Br^- > Cl^- > I^- > NO_3^- > SCN^- > F^- > S_2O_3^{2-} > SO_4^{2-} > CH_3CO_2^-$.

Taking into account their hydration radius (values taken from Imler and Vidaver, 1972: [6]) it can be seen that these ions are classed according to increasing size. There is therefore a relation between the capacity of an anion to stimulate glycine transport and its size: the smaller the anion the greater its efficacy. This relation is illustrated in Fig. 3.

This effect of anions is clearly distinct from that which they exert on the vesicle transmembrane potential. In order to confirm this fact, we studied glucose transport under the same conditions. Glucose transport has no specific anion requirement. Moreover, it is electrogenic [1–3] and may therefore be used to test the effect of anions on the electric potential. These tests may even be performed at a pH of 5.5, as the transport of this sugar still takes place at 60% of its maximum value obtained at pH 7.4.

The results obtained with glucose are different from those reported above for glycine (Fig. 2). The most effective anion in this case is F^-. The SCN^- ion, which strongly stimulates glucose transport at a pH of 7.4 [1–3] only has a moderate action at pH 5.5. The anions may be classed as follows:

$F^- > NO_3^- > Br^- > CH_3CO_2^- > Cl^- > I^- > SCN^- > S_2O_3^{2-} > SO_4^{2-}$

3. Effect of Cl^- on the transport of different concentrations of glycine

This effect of Cl^- was studied at pH 5.5 for glycine concentrations ranging from 50 μM to 10 mM, using vesicles preloaded with K_2SO_4 (50 mM) and then incubated in NaCl (100 mM), Na_2SO_4 (50 mM) or in K_2SO_4 (50 mM). This latter medium gives a value for the diffusion which may then be subtracted from the other measurements in order to calculate the Na^+-dependent transport alone. Short incubation times (15 s) were chosen so that the transport rates could be considered to be sufficiently close to the initial rates. The

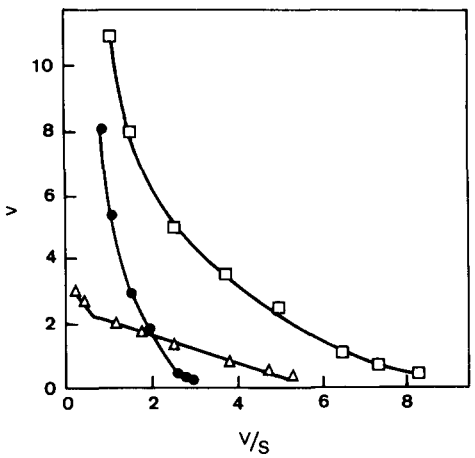


Fig. 4. Kinetic studies of glycine transport at pH 5.5 in the presence of Cl^- (□) and in the absence of Cl^- (●), open triangles show the difference between NaCl and Na_2SO_4. Vesicles are preloaded in K_2SO_4 (50 mM). Diffusion has been subtracted. Results are expressed in Woolf-Hofstee coordinates. v is expressed in μmol/mg protein per 15 s.

results may then be treated like those for enzyme kinetics. Fig. 4 represents the different points obtained on a Woolf-Hofstee plot.

In the presence of NaCl, this curve shows that several components participate in the transport of the amino acid. It may, in fact, be broken down into two straight lines, one characterizing a saturable high-affinity mechanism and the other, a mechanism linearly related to the glycine concentration (Fig. 5). The latter may also correspond to a mechanism with a very low affinity, the K_m of which is probably higher than the highest amino acid concentration used.

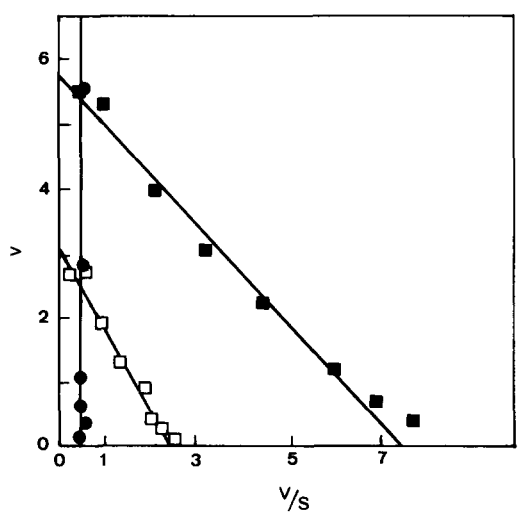


Fig. 5. Na^+-dependent components of glycine transport at pH 5.5: ●, low-affinity component; ■, high-affinity component with Cl^- (K_m = 0.724 mM, V_{max} = 5.54 μmol/mg protein per 15 s; □, high-affinity component in the absence of Cl^- (K_m = 1.202 mM, V_{max} = 2.96 μmol/mg protein per 15 s). Results are expressed in Woolf-Hofstee coordinates. v is expressed in μmol/mg protein per 15 s.

When Cl^- is completely replaced by SO_4^{2-}, the lower the amino acid concentration, the greater the inhibition of glycine transport. This substitution has an unequal effect on the two components of the transport of the amino acid. The very low affinity component is only very slightly modified, whereas the K_m of the mechanism with the high glycine affinity increases and the V_{max} falls (Fig. 5). The reduction in V_{max} is probably more significant than the increase in K_m, taking into account possible inaccuracies in the analysis of the complex curves.

Be that as it may, the specific action on a component with high glycine affinity is confirmed when the values for the amino acid transport rates obtained with NaCl are subtracted from those obtained with Na_2SO_4. On the graph, most of the points fit a straight line as the low-affinity mechanism, which is less affected by Cl^-, is eliminated (Fig. 4: open triangles).

4. Effect of Cl^- on the sodium affinity of the glycine carrier

This effect was looked for by measuring the transport of a very low concentration of glycine (30 μM) in the presence of increasing Na^+ concentrations (from 20 to 100 mM). This was achieved by preloading part of the vesicles in K_2SO_4 (50 mM) and then incubating them in media with different Na_2SO_4 concentrations. The other part was preloaded with KCl (100 mM) and then incubated in different concentrations of NaCl. The osmotic pressure of the incubation media was then adjusted with K_2SO_4 of KCl. Certain measurements were also taken in the absence of sodium to assess the diffusion (replaced by potassium).

The results were plotted according to Woolf and Hofstee with the rate of glycine transport on the Y-axis and the ratio of this transport rate and the Na^+ concentration on the X-axis. Two straight lines are obtained (Fig. 6A), one in the presence

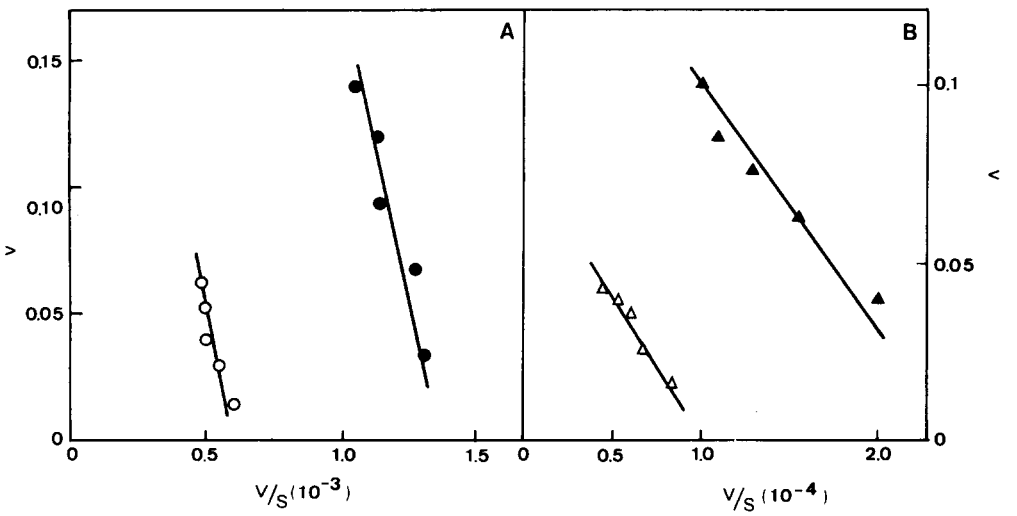


Fig. 6. Effect of Na^+ concentrations on glycine transport (30 μM) at pH 5.5, with Cl^- (●) and without Cl^- (○). (A) Na^+ concentrations ranging from 20 mM to 100 mM. Vesicles are preloaded in K_2SO_4 (50 mM in the case of the experiments with Cl^-), or KCl (100 mM in the case of the experiments with Cl^-). (B) Na^+ concentrations ranging from 100 mM to 500 mM. Vesicles were preloaded in K_2SO_4 (250 mM in the case of the experiments without Cl^-), or KCl (500 mM in the case of the experiments with Cl^-). The isotonicity of the incubation medium is obtained by K_2SO_4 or KCl. Diffusion has been subtracted.
K_m: (A): with Cl^- 370 mM, without Cl^- 301 mM
(B): with Cl^- 266 mM, without Cl^- 394 mM
Results are expressed in Woolf-Hofstee coordinates. v is expressed in μmol/mg protein per 15 s.

and the other in the absence of Cl^-. The slopes of these straight lines are similar. It may therefore be deduced that the K_m of the glycine carrier for sodium is not affected by Cl^- (with Cl^-: 370 mM, without Cl^-: 301 mM). The Na^+ concentrations tested in this experiment are much less than these K_m values, and this may result in a certain inaccuracy in their determination. In order to calculate these K_m more accurately to precisely assess the effect of Cl^-, we used Na^+ concentrations encompassing them, between 100 and 500 mM.

The vesicles were then preloaded with K_2SO_4 (250 mM) or KCl (500 mM) and the results were plotted according to Woolf and Hofstee (Fig. 6B). As with the lower concentrations, two parallel straight lines were obtained with and without Cl^-, confirming the absence of major effect of this anion on the sodium affinity of the carrier. (With Cl^- $K_m = 266$ mM, without Cl^- $K_m = 394$ mM). This experiment also demonstrates an extremely strong effect of the molarity of the medium on the amino acid transport rates. Hence, for the same sodium concentration (100 mM), the glycine transport falls from 122 pm/mg protein, when the vesicles are preloaded with 100 mM KCl, to 20.9 pm/mg protein when the KCl concentration is 500 mM. This is observed, despite the fact that the osmotic pressures of the media inside and outside the vesicles were adjusted. The origin of this phenomenon (modification of the electric potential, antagonist action of potassium and sodium, imperfect equilibration of the vesicles) is currently being investigated.

5. Affinity of Cl^- for the glycine carrier

In order to measure this affinity, vesicles preloaded with K_2SO_4 (250 mM) were incubated in NaCl and Na_2SO_4 in proportions that gave Cl^- concentrations ranging from 20 to 500 mM.

As has been previously observed in the bass with alpha-aminoisobutyric acid, the amino acid carrier has very different affinities for Na^+ and Cl^-. In the case of glycine (30 μM), the Cl^--sensitive mechansim is rapidly saturated and has, for this anion, a much lower K_m than that of sodium (22 mM in comparison with approx. 300 mM for Na^+) (Fig. 7).

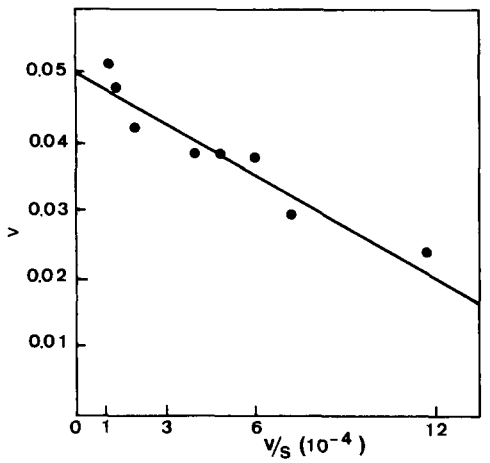


Fig. 7. Effects of Cl^- concentrations on the Na^+-dependent glycine transport (30 μM) at pH 5.5. The Na^+ concentration is 500 mM. Missing Cl^- is replaced by SO_4^{2-} in the incubating medium. Vesicles are preloaded in K_2SO_4 (250 mM). Diffusion has been subtracted. The results are expressed in Woolf-Hofstee coordinates ($K_m = 22$ mM). v is expressed in μmol/mg protein per 15 s.

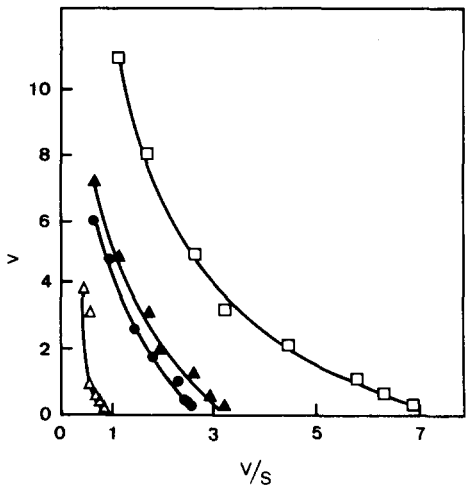


Fig. 8. Kinetic studies of glycine transport at different Na^+ concentrations. □: $Na^+ = 100$ mM, $Cl^- = 100$ mM; ▲: $Na^+ = 25$ mM, $Cl^- = 100$ mM (K^+ replaced missing Na^+); △: $Na^+ = 10$ mM, $Cl^- = 100$ mM (K^+ replaced missing Na^+); ●: $Na^+ = 100$ mM, $SO_4^{2-} = 50$ mM. Vesicles are preloaded with K_2SO_4 (50 mM). Diffusion has been subtracted. Results are expressed as μmol of glycine transported per mg of protein per 15 s, in Woolf-Hofstee coordinates.

6. *Comparative effects of Cl^- and Na^+ on glycine transport*

The following step in this study was to compare the respective effects of Cl^- and Na^+ on the transport of glycine at different concentrations. Transport kinetic studies were therefore carried out either by varying the Na^+ concentration (in the presence of the same Cl^- concentration: 100 mM) or by replacing Cl^- with SO_4^{2-} (in the presence of the same Na^+ concentration: 100 mM). In the first experiment, the Na^+ concentrations (in the form of NaCl) were 10, 25 and 100 mM. Potassium (in the form of KCl) was used instead of sodium to maintain the osmotic pressure. In the second experiment the sodium concentration was 100 mM and SO_4^{2-} replaced Cl^-.

Diffusion was also measured and these values were subtracted from those obtained with sodium salts (Fig. 8).

For a Na^+ concentration of 10 mM (in the presence of 100 mM Cl^-) glycine transport is strongly reduced and the lower the amino acid concentration, the larger the effect. The different points of the Woolf-Hofstee plot fit a curve that corresponds mainly to the very low affinity mechanism.

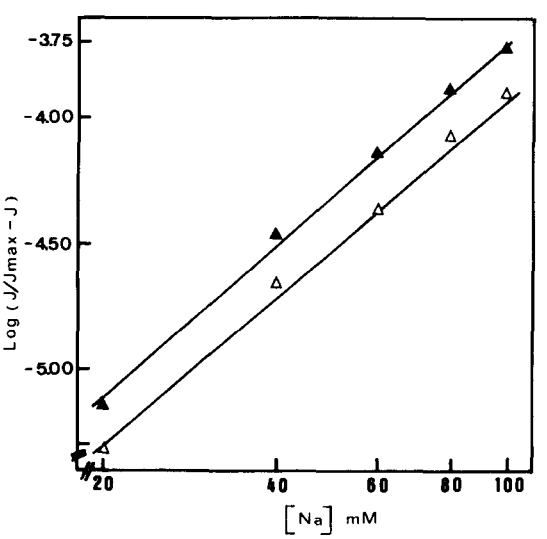


Fig. 9. Effects of Na^+ concentration on glycine transport (30 μM). The results of Fig. 6 have been expressed in logarithmic Hill coordinates (log Na^+ vs. $\log(J/J_{max} - J)$ with: $V_{max} = 5.54$ μmol/mg per 15 s ($+Cl^-$) and 0.96 μmol/mg per 15 s ($-Cl^-$): cf. Fig. 5). ▲, NaCl; △, Na_2SO_4.

The very high affinity mechanism is seen more clearly when the Na^+ concentration increases. The curve obtained with 25 mM Na^+ (and 100 mM Cl^-) is also characterized by the fact that all its points may be superimposed on those obtained with 100 mM Na^+ and in the absence of Cl^- (replaced by SO_4^{2-}).

As far as the apparent effect of Cl^- is concerned, this experiment shows that the presence of this anion on the outside of the vesicles has the same effect on glycine transport as an increase in the Na^+ concentration. Conversely, the replacement of Cl^- by an inert anion such as SO_4^{2-} has the same effect on the carrier as a reduction in the Na^+ concentration.

Discussion

The stimulation of amino acid transport by chloride ions is now an established fact in fish intestines. It also appears that this property is shared by numerous other membrane systems and the same type of Cl^- sensitive amino acid transport mechanism has been identified in, for example, rat nerve cells [7,8], pigeon [3] or human [9] red blood cells and rat brush-border enterocytes [10]. The transport of other molecules is also acheived by Cl^--sensitive mechanisms: serotonin transport in pig blood platelets [11], norepinephrine transport [12], and also that of certain electrolytes such as Na^+ or K^+ [13,14]. In every case, the molecule is normally transported by a Na^+-dependent mechanism (apart from Na^+ transport itself).

(a) General effects of Cl^- on Na^+-dependent membrane transport

In the intestinal brush border of the fishes, the Cl^--dependent mechanism for the transport of neutral amino acids has a maximum at acid pH. Nevertheless, transport still takes place at pH values of up to 8, though the intensity is reduced (Fig. 1). This characteristic optimum pH of amino acid transport in the presence of Cl^- has already been observed in the bass for alpha-aminoisobutyric acid transport [3].

The degree of Cl^--specificity is rather variable and depends on the cell and molecule transported. Most studies show that Br^- is at least equally, if not more effective, than Cl^- for the stimulation of anion-dependent membrane transport systems [6,9,14]. In the bass, this specificity is rather wide as Cl^- may be replaced by a certain number of anions such as F^-, I^- or NO_3^- though the efficacy of these ions is nevertheless lower. Experiments have been carried out on another seafish, the Mediterranean bream (*Boops salpa*), where the anion-specificity is much narrower than in the bass. Only Br^- may effectively replace Cl^- (unpublished results).

In the bass, the glycine carrier has a very strong affinity for Cl^-. The same seems to be true for human and pigeon red blood cells. In human erythrocytes, the glycine transport rate is half its maximum value for a Cl^- concentration of 15 mM [9]. For pigeon red blood cells, the K_m of Cl^- is even lower: 8 mM [6]. In the Madin Darby line of dog kidney cells, the Cl^- concentrations that stimulate cation transport (K^+ as well as Na^+) by up to half of their maximum value, are between 58 and 70 mM [14].

Cl^- has a very variable effect on the kinetic parameters. In the bass it mainly affects one of the two sodium-dependent components of glycine transport, the high affinity mechanism. This result is similar to that obtained in human red blood cells with the same amino acid [9]. It is different, however, from our result in the bass with alpha-aminoisobutyric acid for which both sodium-dependent transport mechanisms were stimulated by Cl^- [3].

As far as the effect of Cl^- on the kinetic constants is concerned, the main effect, and that which has been the most often reported in the literature, is the increase in the V_{max} of the transported molecule [11–14]. This is the case in the bass for glycine (this paper) and for alpha-aminoisobutyric acid [3]. Sometimes, however, a reduction in K_m is observed at the same time. This is the case for glycine and alpha-aminoisobutyric acid transport in the fish intestinal brush-border cells (this paper and Ref. 3), for pig platelet serotonin transport [11].

In pigeon erythrocytes, an increase in the K_m is the major effect of Cl^- on glycine transport [6].

Generally speaking, the Cl^--dependence of membrane transport systems is only partial and the replacement of Cl^- by other anions does not completely inhibit Na^+-dependent transport. This

is shown by the work discussed above, with the exception of that carried out to investigate glycine transport in human erythrocytes which showed that the high-affinity component is completely suppressed in the absence of Cl^- [9].

(b) Mode of action of Cl^- on glycine transport in the bass

In the bass, the affinity of the glycine carrier for Na^+ is not affected in any essential way by Cl^- (Fig. 6) showing that Cl^- does not interfere with the mode of action of Na^+.

This may be confirmed by calculating the number of Na^+ ions that are cotransported per glycine molecule. This number corresponds to the slope of the straight line when log Na is plotted vs. $\log(v/V_{max} - v)$ [17,18]. In the presence, and in the absence of Cl^-, the slope of this line is close to 1 (0.9) (Fig. 9). In order to understand the role of the anion, these findings should be considered in relation with the fact that Cl^- stimulates glycine transport in the same way as an increase in the Na^+ concentration (Fig. 8). The following hypothesis may therefore be formulated: the presence of Cl^- in the incubation medium increases the Na^+ concentration at the glycine transport sites. This may, for example, be a result of a change in the accessibility of the specific carrier sites to Na^+.

A relation between the Na^+ concentration in the incubation medium and the theoretical Na^+ concentration near these sites can be deduced from the data in Fig. 6.

The principle used for this calculation is that the effective Na^+ concentration in the absence of Cl^- is equal to that which provokes the same rate of amino acide uptake in the presence of Cl^-.

$$\frac{K_{m(Na)} + Na_{(i)}}{V_{M(i)} \cdot Na_{(i)}} = \frac{K_{m(Na)} + Na_{(e)}}{V_{M(e)} \cdot Na_{(e)}}$$

where $Na_{(i)}$, Na^+ concentration at the level of the carrier site; $Na_{(e)}$, Na^+ concentration in the incubation medium; $K_{m(Na)}$, $V_{M(i)}$ and $V_{M(e)}$ are the kinetic constants for Na^+ in the absence (e) and in the presence (i) of Cl^-. This gives

$$\frac{1}{Na_{(i)}} = \frac{V_{M(i)}}{V_{M(e)}} \times \frac{1}{Na_{(e)}} + \left(\frac{1}{V_{M(e)}} - \frac{1}{V_{M(i)}}\right) \times \frac{V_{M(i)}}{K_{m(Na)}}$$

which corresponds to a Michaelis-Menten equation plotted after Lineweaver and Burk.

$$\frac{1}{Na_{(i)}} = a \cdot \frac{1}{Na_{(e)}} + b$$

Using the data of Fig. 6

$K_m \simeq 330$ mM, $V_{M(e)} = 234$, $V_{M(i)} = 633$

a K_m is obtained relating the Na^+ concentration at the site of transport to that of the incubation medium of approx. 522 mM.

The effect of Cl^- on the accessibility of Na^+ may be a result of local modifications in the electric charge carried by the transporting system. An effect of Cl^- at a pH 5.5–6 may be explained if, in this case, there was an overall positive charge. This type of action of Cl^- is supported by results obtained in the mammal which show that the effect of the electric potential on glucose transport is related (as in this case for Cl^-) to a change in the local Na^+ concentration [13–16]. In such a mechanism, Cl^- has the advantage that it is smaller than most of the other anions (with the exception of the bromide ion (see Fig. 3)).

Finally, in order to substantiate these hypotheses and throw more light on the role of Cl^- on Na^+-dependent amino acid transport, work is currently being carried out on another marine fish, the Mediterranean bream, *Boops salpa.* In this fish, the transport of amino acids and, in particular, alpha-aminoisobutyric acid [2] is even more dependent on the presence of Cl^- than in the bass [2].

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