Biochimica et Biophysica Acta, 649 (1981) 455–461
Elsevier/North-Holland Biomedical Press

BBA 79475

A CHLORIDE REQUIREMENT FOR Na^+-DEPENDENT AMINO-ACID TRANSPORT BY BRUSH BORDER MEMBRANE VESICLES ISOLATED FROM THE INTESTINE OF A MEDITERRANEAN TELEOST (*BOOPS SALPA*)

GÉRARD BOGÉ and ALAIN RIGAL

Laboratoire Maritime de Physiologie, Institut Michel Pacha, 83500 Tamaris-sur-Mer (France)

(Received April 28th, 1981)

Key words Amino acid transport, Brush border membrane, Na^+ gradient, Cl^- requirement, (Teleost intestine)

The uptake of D-glucose, 2-aminoisobutyric acid and glycine was studied with intestinal brush border membrane vesicles of a marine herbivorous fish: *Boops salpa*. The uptake of these three substances is stimulated by an Na^+ electrochemical gradient ($C_{out} > C_{in}$). For glucose, an increase of the electrical membrane potential generated by a concentration gradient of the liposoluble anion, SCN^-, increases the Na^+-dependent transport. This responsiveness to the membrane potential was confirmed by valinomycin. Differently from glucose, uptake of glycine and 2-aminoisobutyric acid requires, besides the Na^+ gradient, the presence of Cl^- on the external side of the vesicles. In the absence of Cl^-, amino acid uptake is not stimulated by the Na^+ gradient and is not influenced by an electrical membrane potential generated by SCN^- gradient ($C_{out} > C_{in}$) or by a K^+ diffusion potential ($C_{in} > C_{out}$). This Cl^- requirement differs from the Na^+ requirement, since a Cl^- gradient ($C_{out} > C_{in}$) does not result in an accumulation of glycine or 2-aminoisobutyric acid similar to that produced by an Na^+ gradient.

Introduction

In many species of fish, the influence of sodium on intestinal sugar and amino acid transport [1–3] is an important property. Although our knowledge of the mechanism of the effect is not as advanced as in the case of mammals, the essential part played by the brush border membrane has been recognized, as well as the part played by the Na^+ gradient maintained by an $(Na^+ + K^+)$-ATPase sensitive to ouabain [4–6]. It is reasonable to assume in fish, as in mammals, that Na^+ is cotransported with sugars and amino acids, leading to a marked depolarization of the brush border membrane potential [7–9].

In mammals, a new approach for studying the dependence of non-electrolyte transport upon Na^+ was made available following the development of rapid and suitable methods for preparing brush border membranes in a vesicular form [10–12]. Studies carried out with fish likewise showed that it was possible to prepare brush border membrane vesicles suitable for transport studies [13].

This work is intended to specify for an herbivorous mediterranean fish, *Boops salpa*, the properties of the Na^+-dependent transport of a sugar, D-glucose, and two amino acids, 2-aminoisobutyric acid and glycine.

Materials and Methods

Experimental animals

Experiments were carried out with *Boops salpa* (*sparidés*) weighing about 200 g, caught in the Mediterranean Sea near Toulon (Var, France). Fish were kept in the marine aquarium of the laboratory at 15°C ± 1°C for 2 weeks before starting the experiments. They were fed once daily, in the morning, with *Ulva lactuca*, *Cystoseireteum strictae* and *Posidonia oceanica*. The intestine of *Boops salpa* is very long, several times the length of the fish. Aborally several pyloric caeca can be found.

Preparation of vesicles

With *Boops salpa*, the intestine is too fragile for scraping, so the entire intestine was used for the preparation of intestinal brush border membrane vesicles. The method of Malathi et al. [12] as adapted by Crane et al. [13] was used. The tissue was homogenized in 30 vol. of a hypotonic solution of 50 mM mannitol/2 mM Tris-HCl (pH 7.0). 10 mM calcium chloride was added. The homogenate was centrifuged at 9000 × *g* (10 min) instead of 3000 × *g* as mentioned in the original procedure, in order to reduce the contamination by basolateral membranes. The supernatant fluid was then centrifuged at high speed (34 000 × *g*, 20 min). The precipitate, containing brush border membranes, was vesiculated by several successive aspirations through a needle (23-gauge × 1 inch). After addition of mannitol/Tris-HCl equal to the original volume, the vesicles were recovered by high speed as before and resuspended in a small volume of 0.1 M KCl, 0.1 M mannitol, 0.1 M choline chloride or 0.05 M Na_2SO_4 containing Hepes-Tris (5 mM), pH 7 5 and DL-dithiothreitol (1 mM). The composition of media used for loading of the vesicles during preparation is given in the legends to the figures.

Alkaline phosphatase, an enzyme marker for the intestinal brush border membranes [14], was enriched in the extract containing vesicles 13-fold over the initial homogenate (before addition of Ca^{2+}) while ($Na^+ + K^+$)-ATPase an enzyme marker for the baso-lateral membranes which constitute the major contaminent of fish vesicle preparation [13] did not change appreciably during purification (Table I). The total recovery of brush border membranes is nearly 27%, whereas the recovery of the contaminating ($Na^+ + K^+$)-ATPase is only 1.3%.

Uptake method

Incubation was at 25°C and was started by adding 50 µl of the vesicle suspension (protein concentration 2–4 mg/ml) to 250 µl of a solution containing 0.2 mM D-[U-^{14}C]glucose, 2-amino[1-^{14}C]isobutyric acid or [U-^{14}C]glycine, 5 mM Hepes-Tris, pH 7.5, 1 mM DL-dithiothreitol and 0.1 M mannitol or another Na^+ or K^+ salt, the composition of which is given in the legends to the figures. The uptake was stopped by adding an aliquot of 50 µl of the reaction mixture to 1 ml of an ice-cold stop solution (150 mM NaCl). This solution was immediately filtered on 0.22 µm millipore pads and washed with 5 ml of the ice-cold solution. The filters were dissolved in Bray's mixture and the radioactivity remaining on the filters was counted after addition of 'pico-fluor 30'. Transport was expressed as nmol per mg protein found by the method of Lowry et al. [15].

Results

Glucose uptake

Na^+ requirement. During the first min of incubation with NaCl, glucose uptake shows a typical 'overshoot' (Fig. 1b (○)). The accumulation ratio calculated from glucose accumulated in the vesicles at the peak of the overshoot and at equilibrium is approx. 2.5. This overshoot disappears when the Na^+ concentrations inside and outside the vesicles are similar (Fig. 1d (■)) or when Na^+ is replaced by K^+ (Fig. 1e (□)).

Contribution of membrane electrical potential

Glucose uptake is also influenced by the anion associated to Na^+ in the incubation medium. SCN^- is more effective than SO_4^{2-} and Cl^- to stimulate glucose uptake (Fig. 1a (●), b (○) and c (▲)). With SCN^- the

TABLE I

INTESTINAL ENZYME ACTIVITIES OF ALKALINE PHOSPHATASE AND ($Na^+ + K^+$)-ATPase OF HOMOGENATE (H) AND BRUSH BORDER MEMBRANES (M) WITH *BOOPS SALPA*

Determinations were made at 25°C. Values are obtained with a pool of four fish PNP, *p*-nitrophenyl phosphate.

	Homogenate	Membrane	Relative activity (M/H)
Alkaline phosphatase (µmol PNP/mg protein per min)	48	644	13.4
($Na^+ + K^+$)-ATPase (µmol P_i/mg protein per min)	30	20	0 7

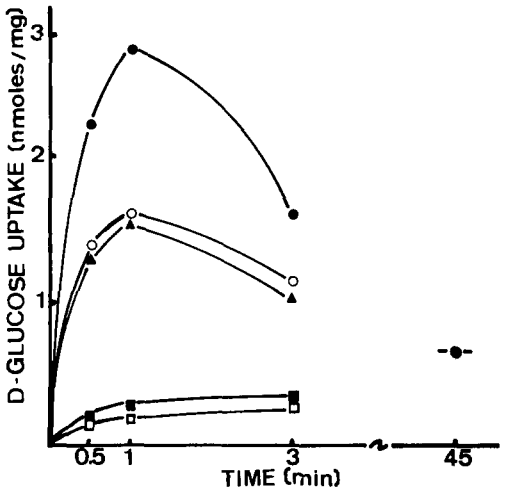

Fig 1 Transport of 0.2 mM D-[U-^{14}C]glucose by intestinal brush border membrane vesicles of *Boops salpa* The experiments present individual but representative observations, they were carried out under following conditions· a, 0 1 M NaSCN outside and 0.1 M mannitol inside the vesicles (•), b, 0.1 M NaCl outside and 0 1 M mannitol inside the vesicles (○), c, 0.05 M Na_2SO_4 outside and 0 1 M mannitol inside the vesicles (▲), d, 0.1 M NaCl + 0 1 M choline chloride outside and inside the vesicles (■); e, 0.1 M KCl outside and 0.1 M choline chloride inside the vesicles (□). Transport assays were performed as described in the text. The equilibrium value (glucose uptake determined after 45 min) is 0 665 ± 0.068 nmol per mg protein.

peak of the overshoot is approx. 1.8-times higher than with Cl^- or SO_4^{2-}. The effects of these anions are generally refered to modifications of the membrane electrical potential [16,17]. They bring evidence of the electrogenic nature of the Na^+-dependent glucose uptake by brush border membrane vesicles of *Boops salpa*. Another convincing evidence is obtained by artificially generating an interior-negative membrane potential by means of a K^+ diffusion potential ($C_{in} > C_{out}$) induced by valinomycin [18]. This potential difference produces a marked increase in the glucose uptake whether the vesicles are incubated in NaCl or in Na_2SO_4 (Table II).

Amino acid uptake

Na^+ requirement. As glucose uptake, 2-aminoisobutyric acid and glycine uptakes show a typical dependence on Na^+. An Na^+ concentration gradient generated by diluting mannitol-loaded vesicles in NaCl produces an overshoot comparable to that previously observed with glucose (Fig. 2a (•)). This accumulation disappears when Na^+ gradient is dissipated (Fig. 2e (■)) or when Na^+ is replaced by K^+ (Fig 2f (□)).

Cl^- requirement. Differently from glucose the Na^+-dependent uptake of glycine and 2-aminoisobutyric acid has a specific requirement for Cl^-. The overshoot produced by the Na^+ gradient can be suppressed when Cl^- is replaced in the incubation medium by SO_4^{2-} or SCN^- (Fig. 2a (•), b (○) and c (▲)).

This Cl^- dependency is apparent when Cl^- is present on the external side of the vesicles. With Cl^- inside only, no overshoot is observed as shown with KCl-loaded vesicles diluted in Na_2SO_4 (Fig. 2d (△)).

When a full Cl^- concentration gradient is imposed by using mannitol-loaded vesicles diluted in NaCl, the Cl^- effect on the amino acid uptake is only slightly enhanced (Fig. 3a (•) and b (○)). In that case, however, the Cl^- concentration gradient may generate an interior-negative membrane potential which may be expected to affect amino acid uptake. The use of K_2SO_4 and KCl (for control)-loaded vesicles incubated in NaCl provides a better means to study the effect of a Cl^- gradient with restricted modifications of the electrical potential. Indeed, SO_4^{2-}

TABLE II

EFFECT OF VALINOMYCIN ON THE 0.2 mM D-[U-^{14}C]-GLUCOSE UPTAKE INTO INTESTINAL BRUSH BORDER MEMBRANE VESICLES OF *BOOPS SALPA*

Vesicles were preloaded with 0.05 M K_2SO_4; they were first incubated for 15 min, at 25°C, with 2% ethanol (−valinomycin) or with 90 μM valinomycin, 2% ethanol (+valinomycin). At zero time a potassium gradient was generated by dilution of vesicles into medium containing 0.05 M Na_2SO_4 or 0.1 M NaCl. Uptake was measured after 30 s as indicated in the text. Values are the average ±S.E. of four assays

Incubation medium	Glucose uptake (nmol/mg protein per 30 s)	
	Na_2SO_4	NaCl
−Valinomycin	1.002 ± 0.119	0.811 ± 0.105
+Valinomycin	2.100 ± 0.289	1.929 ± 0.294
Difference	+109%	+138%

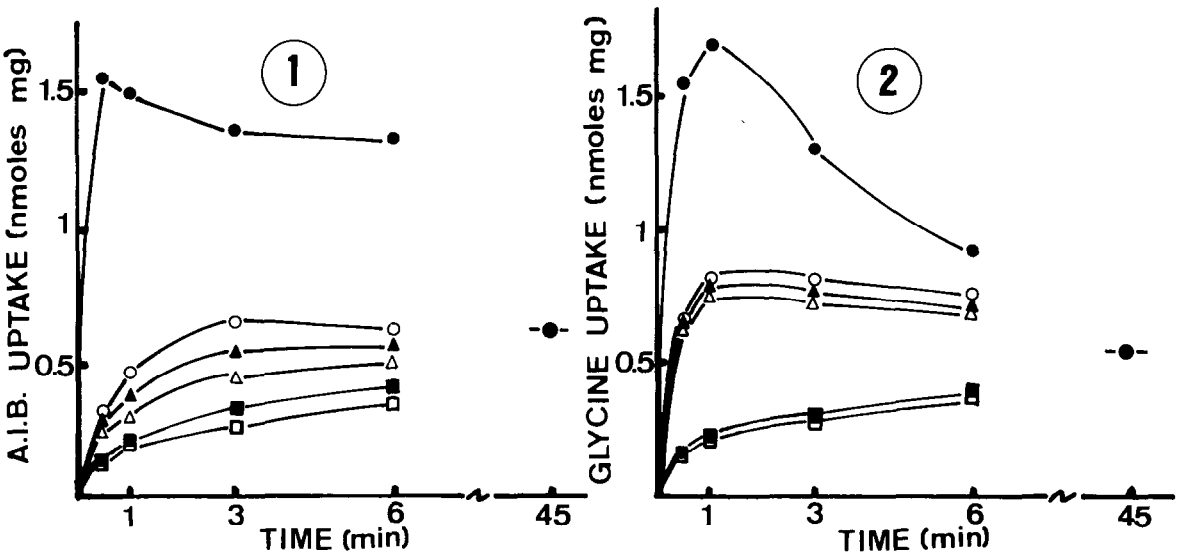

Fig 2 Transport of 0 2 mM 2-amino[1-^{14}C]isobutyric acid (1) and 0.2 mM [U-^{14}C]glycine (2) by intestinal brush border membrane vesicles of *Boops salpa* The experiments present individual but representative observations, they were carried out under following conditions· a, 0 1 M NaCl outside and 0.1 M mannitol inside the vesicles (•); b, 0 1 M NaSCN outside and 0.1 M mannitol inside the vesicles (○), c, 0.05 M Na_2SO_4 outside and 0.1 M mannitol inside the vesicles (▲), d, 0.05 M Na_2SO_4 outside and 0 1 M inside the vesicles (△); e, 0 1 M NaCl + 0.1 M choline chloride outside and inside the vesicles (■); f, 0 1 M KCl outside and 0 1 M choline chloride inside the vesicles (□) Transport assays were performed as described in the text The equilibrium values are 0.604 ± 0 085 nmol/mg protein (2-aminoisobutyric acid uptake determined after 45 min) and 0 630 ± 0 042 nmol/mg protein (glycine uptake determined after 45 min)

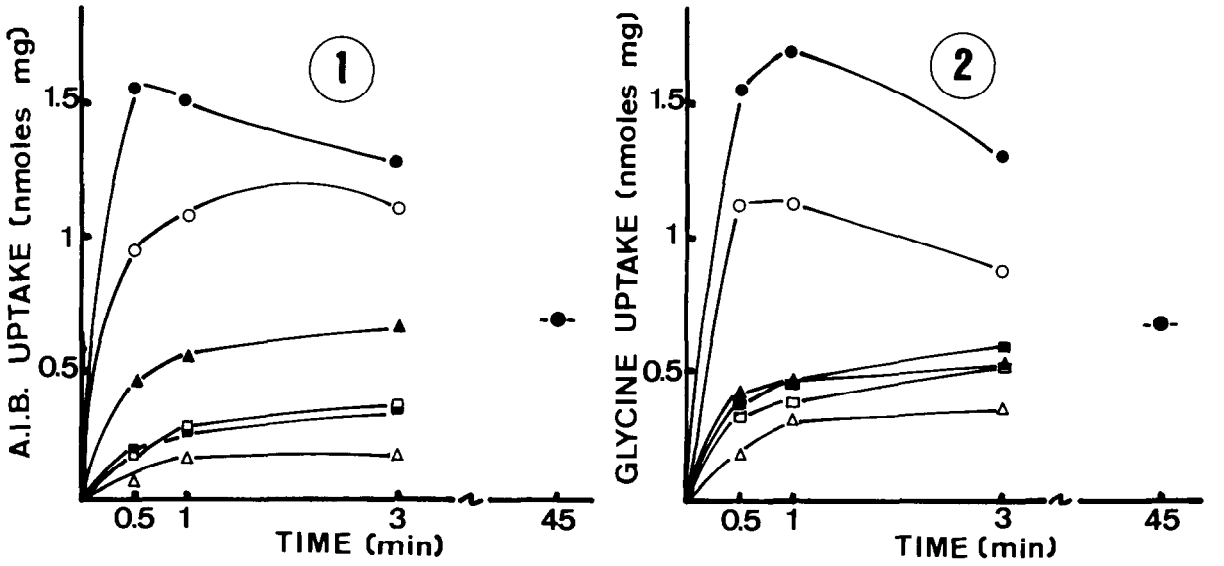

Fig. 3 Effect of a Cl^- gradient on the uptake of 0 2 mM 2-amino[1-^{14}C]isobutyric acid (1) or 0 2 mM [U-^{14}C]glycine (2) into intestinal brush border membrane vesicles of *Boops salpa* The experiments were carried out under different conditions in the presence of an Na^+ gradient· a, Na^+ + Cl^- gradients, 0.1 M NaCl outside and 0 1 M mannitol inside the vesicles (•); b, Na^+ gradient only, 0 1 M NaCl outside and 0 1 M choline chloride inside the vesicles (○), in the absence of an Na^+ gradient c, Cl^- gradient only, 0.1 M outside and 0 05 M Na_2SO_4 inside the vesicles (▲), d, control, 0 05 M Na_2SO_4 outside and inside the vesicles (△); in the absence of Na^+. e, Cl^- gradient only, 0 1 M choline chloride outside and 0.1 M mannitol inside the vesicles (■); f, control, 0.1 M choline chloride outside and inside the vesicles (□). Transport assays present individual but representative observations; they were performed as described in the text The equilibrium values are 0 600 ± 0 057 nmol/mg protein (2-aminoisobutyric acid uptake determined after 45 min) and 0 583 ± 0.059 nmol/mg protein (glycine uptake determined after 45 min)

and Cl^- anions stimulate the glucose uptake of *Boops salpa* vesicles with the same intensity (Fig. 1b (○) and c (▲)) and probably result in comparable electrical potential difference. The Cl^- gradient so obtained causes only an insignificant enhancement of the 30 s amino acid accumulation (2-aminoisobutyric acid: Cl^- gradient = 1.458 nmol/mg protein, control = 1.427 nmol/mg protein; glycine Cl^- gradient = 1.583 nmol/mg protein, control = 1.469 nmol/mg protein.

When the Na^+ gradient is collapsed, or when Na^+ is omitted from the incubation medium, the presence of Cl^- on the external side of the vesicles is not sufficient to energize an overshoot with glycine or 2-aminoisobutyric acid (Fig. 3c (▲), d (△), e (■) and f (□)). At the most, Cl^- gradient permits uptake of 2-aminoisobutyric acid to reach equilibrium more quickly.

Contribution of membrane electrical potential. Contrary to the glucose uptake, the Na^+-dependent uptake of glycine and 2-aminoisobutyric acid by *Boops salpa* vesicles are not electrogenic in the absence of external Cl^-. An increase in the membrane potential produced by an SCN^- gradient (characterized by its effects on glucose uptake. Fig. 1a (●), b (○) and c (▲)) does not stimulate uptakes of glycine or 2-aminoisobutyric acid. A similar result is obtained when the increase in the electrical potential is generated by means of a K^+ diffusion potential. So, the electrical potential produced by valinomycin on K_2SO_4-loaded vesicles diluted in Na_2SO_4 does not stimulate the amino acid uptake (Table III) whereas it does increase glucose uptake (Table II).

In the presence of external Cl^-, the uptake of glycine and 2-aminoisobutyric acid is electrogenic. When K_2SO_4-loaded vesicles and treated by valinomycin are diluted in NaCl, the K^+ diffusion potential stimulates the amino acid uptake (Table III). The response is yet less high than with glucose (Table II) but it is very significant.

Discussion

These results confirm that the general concept of the Na^+ gradient reviewed by Crane [19] can be applied to the transport of glucose by intestinal brush border membrane vesicles of a marine teleost, *Boops salpa*. According to this concept, this transport can be driven by an imposed electrochemical potential gradient of Na^+ which may be described as the sum of electrical component $\Delta\psi$ (the membrane potential, E_m, multiplied by Faraday's constant, F) and of the chemical gradient of Na^+ (expressed as $RT \ln([Na^+_{out}]/[Na^+_{in}])$. However, for amino acid uptake, Cl^- is also required on the external side of membrane vesicles for the stimulation by the Na^+ gradient. With mammals, Cl^- is generally coupled to the transport of electrolytes, such as Na^+ by intestinal brush border membranes [20–22]. With a marine fish, *Cottus scorpius*, Cl^- is also needed for the intestinal exchanges of Na^+ [23]. In the case of mammals, the

TABLE III

EFFECT OF VALINOMYCIN ON THE 0.2 mM 2-AMINO[1-^{14}C]ISOBUTYRIC ACID OR 0.2 mM [U-^{14}C]GLYCINE UPTAKE INTO INTESTINAL BRUSH BORDER MEMBRANE VESICLES OF *BOOPS SALPA*

Vesicles were preloaded with 0.05 M K_2SO_4, they were first incubated for 15 min, at 25°C, with 2% ethanol (–valinomycin) or with 90 μM valinomycin, 2% ethanol (+valinomycin). At zero time a potassium gradient was generated by dilution of vesicles into medium containing 0.05 M Na_2SO_4 or 0 1 M NaCl. Uptake was measured after 30 s as indicated in the text The values are the average ±S.E. of four assays.

Incubation medium	2-Aminoisobutyric acid (nmol/mg protein per 30 s)		Glycine uptake (nmol/mg protein per 30 s)	
	Na_2SO_4	NaCl	Na_2SO_4	NaCl
–Valinomycin	0.268 ± 0.016	1.437 ± 0.076	0.582 ± 0.030	1.637 ± 0.090
+Valinomycin	0.258 ± 0.023	2.171 ± 0 100	0.697 ± 0.072	2.311 ± 0.349
Difference	–4%	+51%	+20%	+41%

transport of non-electrolytes by intestinal brush border membranes does not show such a Cl^- requirement It has, however, been observed in other kinds of membrane such as the membranes of rat brain where Cl^- is required for the transport of γ-amino-*n*-butyric acid [24] or proline [25], such as pigeon red cell membranes where Cl^- stimulates the glycine uptake [26], and such as human platelet membranes where the 5-hydroxytryptamine uptake is also influences by Cl^- [27, 28].

In the case of glycine uptake by pigeon erythrocytes, Imler and Vidaver [26] have suggested that Cl^- may take part in the formation of the transported complex but probably does not energize the transport Cl^- has been also postulated to be an essential component of the translocated complex for the transport of 5-hydroxytryptamine by plasma membrane vesicles isolated from human blood platelets [28]. According to Rudnick and Nelson [28] the amine uptake is electroneutral, since valinomycin added to potassium-loaded vesicles does not stimulate the uptake. As such Na^+- and Cl^--linked transport is no longer sensitive to electrical potential changes, it appears that Cl^- may be contransported together with Na^+ and that it drives the solute transport.

With *Boops salpa*, the possibility that Cl^- may contribute to the energetics of the glycine or 2-aminoisobutyric acid transport process appears to be excluded by the quoted observation that in the absence of Na^+ gradient, a Cl^- gradient alone is not able to drive the amino acid accumulation in the vesicles, as shown with Na_2SO_4 preloaded vesicles incubated with NaCl (Fig. 3 c (▲)). However, if the permeability of the vesicles to Cl^- is very large relative to that of Na^+, the Cl^- gradient may be dissipated rapidly and its effects on uptake of glycine and 2-aminoisobutyric acid may be more transient than those of the Na^+ gradient. So, it is probable that under the experimental conditions, Cl^- would influence the amino acid uptake without energizing it, whereas the Na^+ gradient alone would drive the overshoot of accumulation. The amino acid uptake would then take place together with the translocation of positive charges because transport of glycine and 2-aminoisobutyric acid is sensitive to an increase of the electrical potential (inside negative). If Cl^- is contransported with Na^+, a possibility which cannot be excluded, it is likely that more Na^+ than Cl^- would be transported as for glycine transport by pigeon erythrocytes [26]. Moreover, the higher response of glucose transport than amino acid transport to an imposed electrical potential difference (Tables II and III), may be an indication that uptake of glucose, glycine and 2-aminoisobutyric acid exhibit a different Na^+ solute stoichiometry, as suggested by the observations of Kimmich and Randles [29].

Finally, although it is not yet possible to know if Cl^- energizes or not the uptake of glycine and 2-aminoisobutyric acid, the presence in the intestine of fish of such a Cl^- linked amino acid transport seems interesting with regard to the knowledge of transport mechanism in Vertebrates, and because such a process seems absent from the intestine of more advanced species

Acknowledgement

The authors wish to thank Professeur Robert K. Crane for his advice and Professeur Gabriel Pérès for his generous support and encouragement.

References

1 Read, C.P (1967) Biol. Bull. 133, 630–642
2 Bogé, G , Rigal, A and Pérès, G. (1977) Ann Biol Anim. Bioch Biophys. 17, 637–643
3 Smith, R.L. (1969) Comp. Biochem. Physiol 30, 1115–1123
4 Smith, M.W. (1964) Experientia 20, 613–614
5 Cartier, M., Buclon, M and Robinson, J.W L (1979) Comp. Biochem Physiol 62A, 537–541
6 Bogé, G , Rigal, A and Pérès, G (1979) Comp Biochem Physiol 64A, 537–541
7 Smith, M W. (1966) J Physiol. 184, 559–573
8 Mepham, T.B and Smith, M W (1966) J Physiol 184, 673–684
9 Albus, H and Siegenbeek Van Heukelom, J. (1976) Comp. Biochem Physiol 54A, 113–119
10 Schmitz, J., Preiser, H., Maestracci, D , Gosh, B.K , Cerda, J J. and Crane, R K (1973) Biochim Biophys Acta 323, 98–112
11 Kessler, M , Acuto, O., Storelli, C , Murer, H , Muller, M and Semanza, G (1978) Biochim. Biophys Acta 506, 136–154
12 Malathi, P , Preiser, H., Fairclough, C.P , Mallet, M and Crane, R.K (1979) Biochim Biophys Acta 554, 259–263
13 Crane, R K., Bogé, G. and Rigal, A (1979) Biochim. Biophys. Acta 554, 264–267
14 Eichholz, A (1967) Biochim Biophys Acta 135, 475–482

15 Lowry, O.H , Rosebrough, N.J , Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
16 Lever, J (1977) Biochemistry 16, 4328–4334
17 Sigrist-Nelson, K., Murer, H. and Hopfer, U. (1975) J Biol. Chem. 250, 5674–5680
18 Pressman, B C (1976) Annu Rev. Biochem. 45, 501–530
19 Crane, R.K (1977) Rev Physiol. Biochem. Pharmacol 78, 99–159
20 Nellans, H.N., Frizzell, R.A. and Schultz, S.G (1973) Am. J. Physiol. 225, 467–475
21 Nellans, H.N., Frizzell, R A. and Schultz, S G (1974) Am. J. Physiol 226, 1131–1141
22 Frizzell, R.A., Nellans, H.N , Rose, R C., Marksheid-Kaspi, L. and Schultz, S.G (1973) Am. J. Physiol. 224, 328–337
23 House, C.R and Green, K (1965) J. Exp. Biol. 42, 177–189
24 Kanner, B I. (1978) Biochemistry 17, 1207–1211
25 Kanner, B.I. and Sharon, I (1980) Biochim. Biophys. Acta 600, 185–194
26 Imler, J R and Vidaver, G A (1972) Biochim. Biophys. Acta 288, 185–194
27 Rudnick, G (1977) J Biol. Chem. 252, 2170–2174
28 Rudnick, G. and Nelson, P J (1978) Biochemistry 17, 4739–4742
29 Kimmich, G.A and Randles, J. (1980) Biochim. Biophys Acta 596, 439–444