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ANALYSIS OF TWO CHLORIDE REQUIREMENTS FOR SODIUM-DEPENDENT AMINO ACID AND GLUCOSE TRANSPORT BY INTESTINAL BRUSH-BORDER MEMBRANE VESICLES OF FISH

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Intestinal brush border vesicles of a Mediterranean sea fish (*Dicentrarchus labrax*) were prepared using the Ca^{2+}-sedimentation method. The transport of glucose, glycine and 2-aminoisobutyric acid is energized by an Na^+ gradient (out > in). In addition, amino acid uptake requires Cl^- in the extravesicular medium (2-aminoisobutyric acid more than glycine). This Na^+- and Cl^--dependent uptake is electrogenic, since it can be stimulated by negative charges inside the vesicles. The specific Cl^- requirement of glycine and 2-aminoisobutyric acid transport is markedly influenced by pH, a change from 6.5 to 8.4 reducing the role played by Cl^-. In the presence of Cl^-, the K_m of 2-aminoisobutyric acid uptake is reduced and its V_{max} is enhanced. Cl^- affects also a non-saturable Na^+-dependent component of this amino acid uptake. Amino acid transport is also increased by intravesicular Cl^- (2-aminoisobutyric acid less than glycine). This effect is more concerned with glucose uptake, which can be then multiplied by 2.3. A concentration gradient (in > out) as well as the presence of Na^+ in the incubation medium seems to enter into this requirement. This intravesicular Cl^- effect is not influenced by pH between 6.5 and 8.4.

Introduction

In fish, transport of sugar and amino acid is driven by the Na^+ electrochemical gradient. This concept has been clarified in vivo [1] with swimming fish, and in vitro with isolated intestines [2–4] or brush-border membrane vesicles [5]. In addition, recent work has demonstrated a Cl^--dependent uptake of amino acid transport in a herbivorous fish, *Boops salpa*, Cl^- being necessary together with Na^+ for the 'uphill' transport of glycine and 2-aminoisobutyric acid [6]. The object of this work was first to give more extensive insight into this Cl^- requirement in fish. For this purpose, the anionic dependence of amino acid transport in a carnivorous fish (*Dicentrarchus labrax*) was analysed. Two effects of Cl^- were found, one concerning Cl^- in the intravesicular medium, the other being related to extravesicular Cl^-. The object of this work was also to give deeper insight into the different mechanisms concerned with these Cl^- requirements in fish by disclosing their principal characteristics with regard to Cl^- or amino acid concentrations or to pH.

Materials and Methods

Experimental animals

Sea bass (*D. labrax*), weighing about 150 g were reared in a center of C.N.E.X.O. (Centre National pour l'Exploitation des Océans) at Palavas les Flots (France) in outdoor ponds under natural climatic conditions. They were kept at

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15°C ± 1°C in the marine aquarium of the laboratory for 2 weeks before the start of the experiments. They were fed daily with commercial sea bass food pellets. The intestine of *Dicentrarchus* is a tube folded back three times on itself with five pyloric caeca on the anterior part. The structure and the development of its digestive tract were recently described by Benhalima [7].

Preparation of vesicles

Intestinal brush-border membranes were isolated by the Ca^{2+}-aggregation method previously described [5]. The preparation of vesicles was begun by scraping the mucosa from the total intestine. The membranes obtained were suspended in a medium of 5 mM Hepes-Tris, (pH 7.4), 1 mM DL-dithiothreitol and 100 mM mannitol or another K^+ or Na^+ salt. Details of the nature of salt used are given in the legends to the figures and tables.

Enzyme assays

The state of purity of the membranes was evaluated by the enrichment of enzymes specific for the intestinal brush border membranes; alkaline phosphatase activity was determined by the method of Eichholz [8], maltase, sucrase and trehalase activities by the method of Lloyd and Whelan [9] and Leucyl naphthylamidase activity by the method of Goldbarg and Rutenberg [10]. The contamination by basolateral membranes, mitochondria and microsomes was evaluated by, respectively, $(Na^+ + K^+)$-ATPase activity [11], succinate dehydrogenase activity [12] and NADPH cytochrome *c* reductase activity [13]. All determinations were carried out at 37°C and expressed per min and per g protein determined by the method of Lowry et al. [14] with bovine serum albumin as standard.

Transport measurements

The uptake of radioactively labelled solutes was measured at 25°C by a rapid filtration technique. 50 μl medium containing vesicle suspension (protein concentration 2–4 mg/ml) were incubated in 250 μl of a solution of 0.2 mM D-[U-^{14}C]glucose, 2-amino[1-^{14}C]isobutyric acid or [U-^{14}C]glycine and 100 mM mannitol or another Na^+ or K^+ salt (see legends). 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). The membranes were immediately collected on 0.22 μm Millipore pads and washed with 5 ml of the ice-cold solution. Filters were dissolved in Bray's mixture and, after addition of 10 ml scintillation fluid "Pico fluor 30", radioactivity was counted in a Packard Tricarb scintillation counter. Transport was expressed as nmol per mg protein determined as previously [14].

Results

Evaluation of the membrane vesicle preparation

The measurement of maltase, alkaline phos-

TABLE I

ENZYME PROFILE OF ISOLATED BRUSH BORDER MEMBRANES OF *D. LABRAX*

The enzyme activities are measured at 37°C. All activities are expressed as μmol/g protein per min except for NADPH cytochrome *c* reductase, which is expressed as ΔA/g protein per min. Values are means ± S.E. of four animals. n.d., not detectable.

	Homogenate (H)	Membrane (M)	Relative activity (M/H)
Protein (mg)	185 ± 22	5.2 ± 0.9	0.028
Alkaline phosphatase	248 ± 14	2297 ± 125	9.26
Maltase	298 ± 21	2842 ± 215	9.54
Sucrase	12.9 ± 0.4	159 ± 23	12.3
Trehalase	53.1 ± 2.5	492 ± 74	9.27
Leucyl naphthylamidase	165 ± 7	1342 ± 238	8.13
Succinate dehydrogenase	14.2 ± 1.0	1.77 ± 0.06	0.13
NADPH cytochrome *c* reductase	66.7 ± 8	n.d.	–
$(Na^+ + K^+)$-ATPase	37 ± 5	47 ± 9	1.27

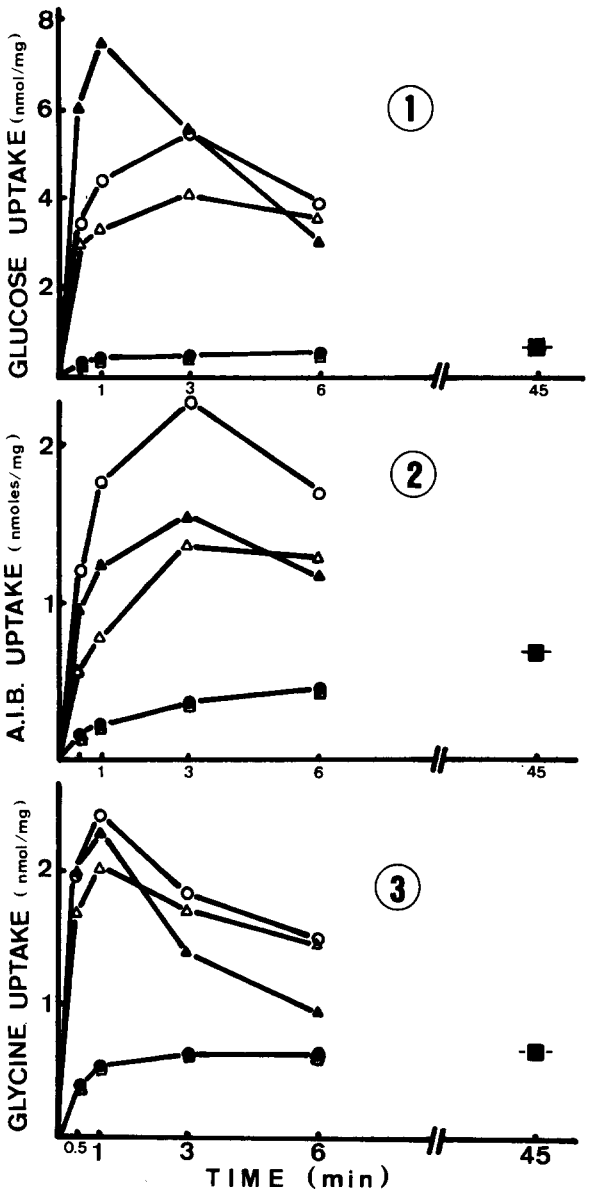

Fig. 1. Time course of 0.2 mM D-[U-^{14}C]glucose (1), 0.2 mM 2-amino[1-^{14}C]isobutyric acid (AIB) (2) and 0.2 mM [U-^{14}C]glycine (3) uptake by intestinal brush-border membrane vesicles of *D. labrax*. These experiments present individual but representative observations. Vesicles were preloaded with 100 mM mannitol and incubated in the presence of 50 mM Na_2SO_4 (△), 100 mM NaSCN (▲), 100 mM NaCl (○), 100 mM KCl (●) or 50 mM K_2SO_4 (□). Transport assays were performed as described in the text. The equilibrium values are 0.630 ± 0.068 nmol/mg protein (glucose uptake determined after 45 min), 0.667 ± 0.067 nmol/mg protein (2-aminoisobutyric acid uptake determined after 45 min), 0.663 ± 0.053 nmol/mg protein (glycine uptake determined after 45 min). Each experiment was performed on at least three separate fresh membrane preparations.

phatase and leucyl naphthylamidase activities in the initial homogenate (before the addition of calcium) and the brush-border preparation (Table I) shows that the vesicles are pure enough to be used for transport studies; the enrichment in specific activity (final pellet/homogenate) was virtually the same for the luminal membrane markers chosen and comparable to the degree of purification previously obtained with *Scyliorhinus canicula* [5]. About 25% of the brush border membranes were recovered. Contamination by the other membranes was relatively small or not detectable, since succinate dehydrogenase, NADPH cytochrome *c* reductase and ($Na^+ + K^+$)-ATPase activities were either reduced or poorly enriched in the brush-border preparation.

Role of extravesicular chloride in amino acid transport

Characterization of chloride-dependent amino acid transport. Glycine and 2-aminoisobutyric acid transport by brush-border membrane vesicles of *D. labrax* has a typical dependence for Na^+ which resembles that observed with glucose (Fig. 1). In addition there is an influence of the anion associated with Na^+ in the incubation medium which is different for glucose and for amino acids. Na^+-dependent glucose transport is more stimulated by SCN^- than by Cl^- or SO_4^{2-} gradients. In contrast, Na^+-dependent 2-aminoisobutyric acid uptake is more effective when Na^+ is associated with Cl^- than with SCN^- or SO_4^{2-}. With glycine, the effects of Cl^- and SCN^- are equivalent and give better stimulation than SO_4^{2-}. When the anion concentrations inside and outside the vesicles are similar, thus reducing the effect of these anions on the electrical potential difference across the membrane, the overshooting uptake of glycine and 2-aminoisobutyric acid is reduced with SO_4^{2-} and SCN^- but not with Cl^- (Fig. 2).

Cl^- has no specific effect when Na^+ is omitted from the incubation medium and replaced by K^+ (Fig. 1).

Influence of membrane potential. An interior-negative potential has been generated on vesicles preloaded with K_2SO_4 by addition of valinomycin followed by incubation in NaCl or Na_2SO_4. This potential brings about an enhancement of amino acid transport with Cl^- as with SO_4^{2-}. Expressed

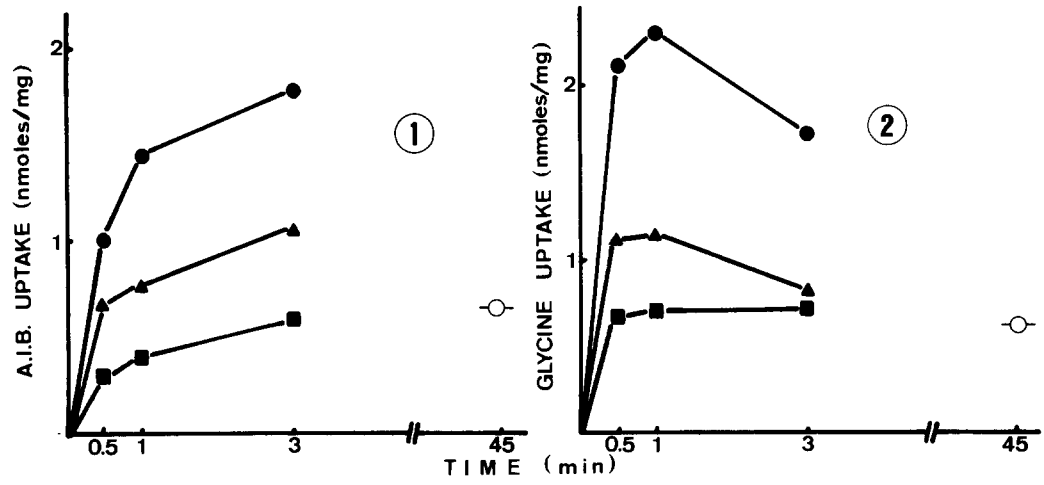

Fig. 2. Transport of 0.2 mM 2-amino[1-^{14}C]isobutyric acid (1) and 0.2 mM [U-^{14}C]glycine (2) uptake by intestinal brush-border membrane vesicles of *D. labrax* in the absence of anion gradient. The experiments were carired out under different conditions: 100 mM NaCl outside and 100 mM KCl inside the vesicles (●), 100 mM NaSCN outside and 100 mM KSCN inside the vesicles (▲), 50 mM Na_2SO_4 outside and 50 mM K_2SO_4 inside the vesicles (■). The equilibrium values (○) are 0.637 ± 0.058 nmol/mg protein (2-aminoisobutyric acid uptake after 45 min), 0.661 ± 0.053 nmol/mg protein (glycine uptake after 45 min). Each experiment was performed on at least three separate fresh membrane preparations. The figures are presented as individual but representative observations.

as percentage of control, this stimulation is comparable with the two anions (Table II). Under these high-potential conditions, the effect of Cl^- on 2-aminoisobutyric acid transport persists and is always more marked than with glycine. If the differences between NaCl and Na_2SO_4 are calculated, an effect of the inside-negative potential on glycine uptake, but above all on 2-aminoisobutyric acid uptake, is observed. So, the chloride-dependent amino acid uptake by the intestinal brush-border membrane vesicles of *D. labrax* can be established as an electrogenic transport process.

Influence of chloride concentration. Under a constant Na^+ concentration (100 mM), the stimulation of the amino acid uptake by Cl^- is attenuated when the anion concentration increases. In contrast, increasing Na^+ concentration (under a constant Cl^- concentration: 100 mM) leads to a linear stimulation. This phenomenon has been observed for 2-aminoisobutyric acid uptake with

TABLE II

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 *D. LABRAX*

Vesicles were preloaded with 50 mM K_2SO_4; they were first incubated for 15 min at 25°C with 2% ethanol (control) or with 90 μM valinomycin, 2% ethanol (Valinomycin). At zero time, a potassium gradient was generated by dilution of vesicles into medium containing 50 mM Na_2SO_4 or 100 mM NaCl. Uptake was measured after 30 s. The values are average ± S.E. of four assays.

Incubation medium	2-Aminoisobutyric acid uptake (nmol/mg protein per 30 s)			Glycine uptake (nmol/mg protein per 30 s)		
	Na_2SO_4	NaCl	NaCl − Na_2SO_4	Na_2SO_4	NaCl	NaCl − Na_2SO_4
Control	0.406 ± 0.052	1.271 ± 0.196	0.865	1.266 ± 0.215	1.449 ± 0.281	0.183
Valinomycin	0.734 ± 0.076	2.134 ± 0.466	1.400	3.007 ± 0.255	3.294 ± 0.260	0.287
Difference (%)	+81	+68	+62	+137	+127	+57

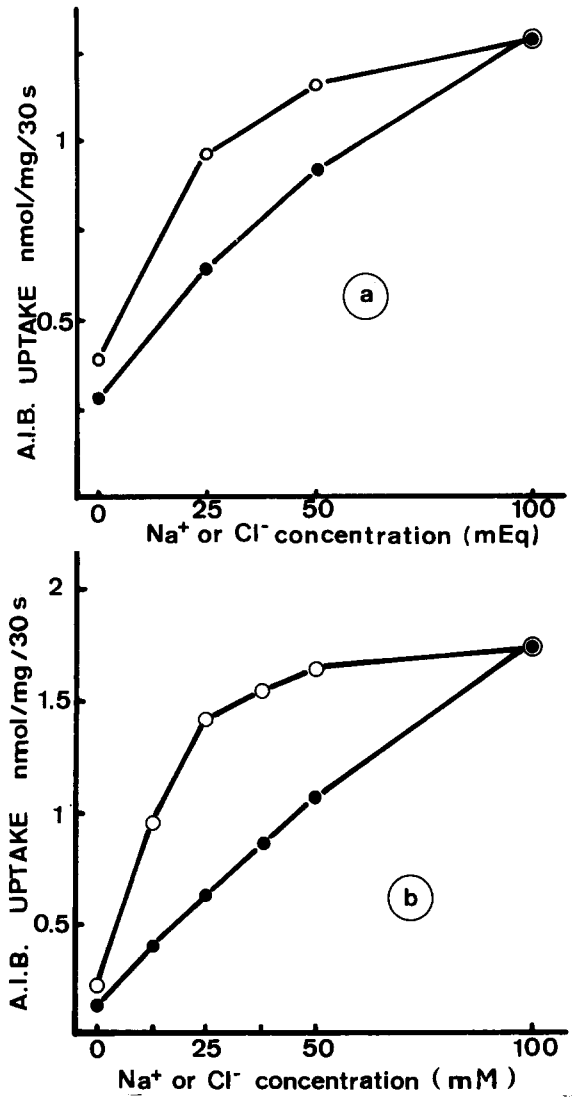

Fig. 3. Effect of Cl^- and Na^+ concentrations on 0.2 mM 2-amino[1-^{14}C]isobutyric acid uptake by intestinal brush border membrane vesicles of *D. labrax* (a) or of *B. salpa* (b). The Cl^- effects (○) are measured at constant Na^+ concentration (100 mM; NaCl is replaced by Na_2SO_4). The Na^+ effects (●) are measured under constant Cl^- concentration (100 mM; NaCl is replaced by choline chloride). Each plot is a mean of four experiments.

vesicles of *D. labrax* (Fig. 3a), but is still more clear with vesicles of an herbivorous fish *Boops salpa* (Fig. 3b). Consequently, at constant NaCl concentration, the amino acid uptake will be limited above all by the lower sensitivity of the transport process to Na^+ than to Cl^-.

Influence of pH. Further studies have revealed that the role played by Cl^- in amino acid transport tends to be reduced at basic pH (Fig. 4). This property was studied at pH 6.5, 7.4 and 8.4 with mannitol-preloaded vesicles buffered at the pH of the incubation medium. The results clearly show that 2-aminoisobutyric acid and glycine uptake is greatly stimulated by Cl^- at pH 6.5 but not at pH 8.5. When Na^+ is replaced in the incubation medium by K^+, low levels of amino acid uptake are found, and no influence of Cl^- exists between pH 6.5 and 8.4 (data not shown).

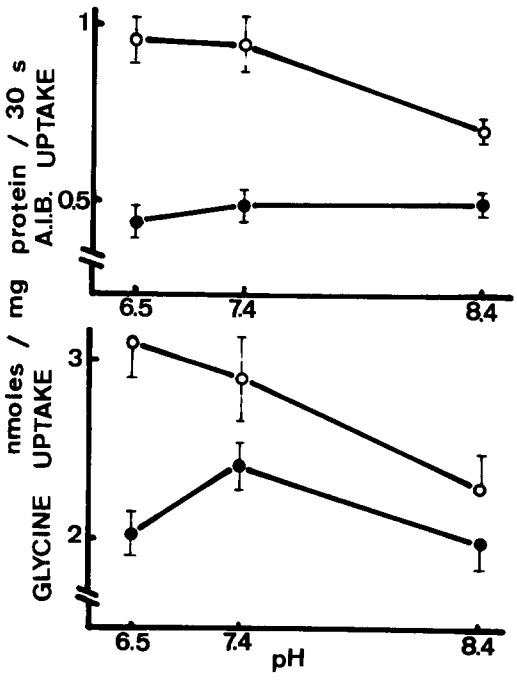

Fig. 4. Effect of pH on 0.2 mM 2-amino[1-^{14}C]isobutyric acid or 0.2 mM [U-^{14}C]glycine uptake by intestinal brush-border membrane vesicles of *D. labrax*. Experiments were carried out on vesicles preloaded with 100 mM mannitol and buffered at the pH of the incubation medium. Each vesicle preparation was assayed for the transport of the two solutes at pH 6.5, 7.4 and 8.4 with 100 mM NaCl (○) or 50 mM Na_2SO_4 (●) in the incubation medium. At the three pH values, differences between NaCl and Na_2SO_4 in the transport of amino acids are all statistically significant using the *P* paired test. Six animals were used for each experiment.

Influence of amino acid concentrations. The Cl^- requirement of a broad range of 2-aminoisobutyric acid concentrations has been investigated using short-duration incubations (15 s) with NaCl, Na_2SO_4 and K_2SO_4. The results obtained with NaCl and Na_2SO_4 were then corrected for Na^+-independent transport with K_2SO_4 and plotted as in Fig. 5. These plots reveal two components of

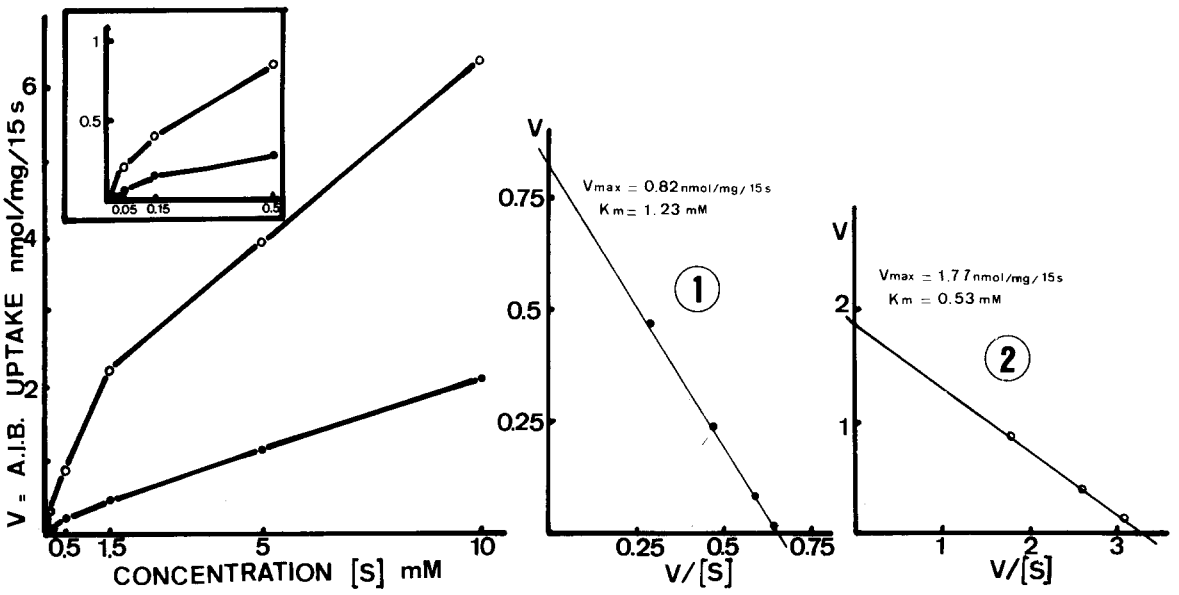

Fig. 5. Kinetics of 2-amino[1-^{14}C]isobutyric acid transport by intestinal brush-border membrane vesicles of *D. labrax*. Vesicles were preloaded with 100 mM mannitol and incubated for 15 s at pH 7.4 in the presence of 100 mM NaCl or 50 mM Na_2SO_4 or 50 mM K_2SO_4. Results with K_2SO_4 were subtracted from values with NaCl or Na_2SO_4 to calculate the specific Na^+-dependent 2-aminoisobutyric acid uptake with (○) or without (●) Cl^-. The saturable part of the curves was resolved using Eadie-Hofstee coordinates in the presence of Na_2SO_4 (1) or NaCl (2). Each plot represents a mean of four animals.

Na^+-dependent 2-aminoisobutyric acid uptake: a saturable component for the transport of small concentrations (0.05 to 1.5 mM) and a non-saturable component (up to 10 mM). The saturable process obeys simple Michaelis-Menten kinetics, the constants of which are influenced by the anion accompanying Na^+: Cl^- results in an increase in the V_{max} and a decrease in the K_m. The linear component also reveals differences related to the nature of anion: the slope is higher with Cl^- than with SO_4^{2-} (Fig. 5).

Role of intravesicular chloride on amino acid and glucose transport

Characterization of stimulation of amino acid and glucose transport by intravesicular chloride. The transport of amino acids by intestinal brush border membrane vesicles of *D. labrax* can be stimulated when the vesicles are preloaded with KCl instead of K_2SO_4 (Table III). This has been shown using Na_2SO_4 in the incubation medium. This stimulation partially results from the low external Cl^- concentration (17 mM) added with KCl preloaded vesicles, since it is attenuated when the same Cl^- concentration is added as choline chloride with K_2SO_4 preloaded vesicles. Under these conditions, stimulation persists with KCl-preloaded vesicles (principally on glycine uptake), which can be attributed to the presence of Cl^- inside the vesicles.

This effect of Cl^- inside has also been found with glucose transport (Table IV), which is 2.3-fold higher when vesicles incubated in Na_2SO_4 are preloaded with KCl instead of K_2SO_4. Contrary to the preceding observations with amino acids, the addition to the control of choline chloride in the incubation medium to balance the Cl^- added with KCl preloaded vesicles is ineffective in changing the intensity of this phenomenon.

Role of chloride gradient. The stimulation of amino acids and glucose transport by internal Cl^- appears to proceed from a Cl^--inverse gradient ($Cl^-_{in} > Cl^-_{out}$), since it is attenuated when external Na_2SO_4 is replaced by NaCl (Tables III and IV). For glucose (Table IV) a residual Cl^- effect persists owing to possible imperfect Cl^- equilibration.

Evaluation with time of internal chloride requirement. Contrary to the effect of external Cl^-, which is already intense after 15 s incubation (about +240% with NaCl as compared to Na_2SO_4 in the incubation medium), the internal Cl^- effect, shown

TABLE III

INTRAVESICULAR AND EXTRAVESICULAR Cl^- EFFECTS ON 0.2 mM 2-AMINO[1-^{14}C]ISOBUTYRIC ACID OR 0.2 mM [U-^{14}C]GLYCINE UPTAKE MEASURED IN THE PRESENCE OF AN Na^+ GRADIENT

Vesicles were preloaded with 50 mM K_2SO_4 or 100 mM KCl and incubated as described in Materials and Methods in the presence of 50 mM Na_2SO_4 or 100 mM NaCl. For experiments with vesicles preloaded with K_2SO_4 and incubated in Na_2SO_4, choline chloride was added to a final concentration of 17 mM. Uptake was measured after 30 s at 35°C as indicated in the text. The values are average ± S.E. of four assays (the values of intravesicular spaces are given in Table IV for glucose). CC, choline chloride.

Vesicles preloaded in:	Vesicles incubated in:	2-Aminoisobutyric acid transport (nmol/mg protein per 30 s)			Glycine transport (nmol/mg protein per 30 s)		
		Na_2SO_4	Na_2SO_4 + 17 mM CC	NaCl	Na_2SO_4	Na_2SO_4 + 17 mM CC	NaCl
K_2SO_4		0.346 ±0.050	0.724 ±0.153	1.675 ±0.295	0.932 ±0.050	1.053 ±0.073	1.486 ±0.307
KCl		0.908 ±0.151	–	1.500 ±0.206	2.009 ±0.327	–	1.835 ±0.076

in Tables III and IV, is not very marked during the early period of incubation. So, with KCl-preloaded vesicles, the enhancement of glucose transport is only of 29% after 15 s, whereas after 45 s it reaches 63% (Fig. 6).

Influence of pH. As previously, the influence of pH was studied with vesicles buffered at the pH of the incubation medium between 6.5 and 8.4. Vesicles were preloaded with 100 mM KCl and incubated in the presence of 100 mM NaCl (as control) or 50 mM Na_2SO_4 to set up the conditions of an inverse Cl^- gradient (in > out). Glucose was chosen as test molecule because of the greatest sensitivity of its transport to internal Cl^-. Contrary to the effect of external Cl^- on amino acids, the effect of internal Cl^- on glucose transport is not influenced by pH between 6.5 and 8.4, the curves obtained with internal na_2SO_4 and NaCl at the three pH values being parallel (Fig. 7).

Effect of Na^+ on internal Cl^- requirement. The effect of internal Cl^- requires the presence of Na^+. When Na_2SO_4 and NaCl are substituted by K_2SO_4

TABLE IV

INTRAVESICULAR AND EXTRAVESICULAR Cl^- EFFECTS ON 0.2 mM D-[U-^{14}C]GLUCOSE UPTAKE MEASURED IN THE PRESENCE OR THE ABSENCE OF Na^+ BY INTESTINAL BRUSH BORDER MEMBRANES OF *D. LABRAX*

Vesicles were preloaded with 50 mM K_2SO_4 or 100 mM KCl and incubated in the presence of 50 mM Na_2SO_4, 100 mM NaCl, 50 mM K_2SO_4 or 100 mM KCl. For experiments with vesicles preloaded in K_2SO_4 and incubated in Na_2SO_4, choline chloride was added to a final concentration of 17 mM. Uptake was measured after 30 s at 25°C. The values are average ± S.E. of four assays. The equilibrium values are 0.520 nmol/mg protein determined after 45 min with KCl preloaded vesicles incubated in NaCl and 0.532 nmol/mg protein determined after 45 min with K_2SO_4 preloaded vesicles incubated in NaCl.

Vesicles preloaded in:	Vesicles incubated in:	Glucose transport (nmol/mg protein per 30 s)				
		Na_2SO_4	Na_2SO_4 + 17 mM CC	NaCl	K_2SO_4	KCl
K_2SO_4		1.949 ±0.217	1.970 ±0.198	2.028 ±0.480	–	–
KCl		4.503 ±0.521	–	3.022 ±0.088	0.330 ±0.040	0.274 ±0.017

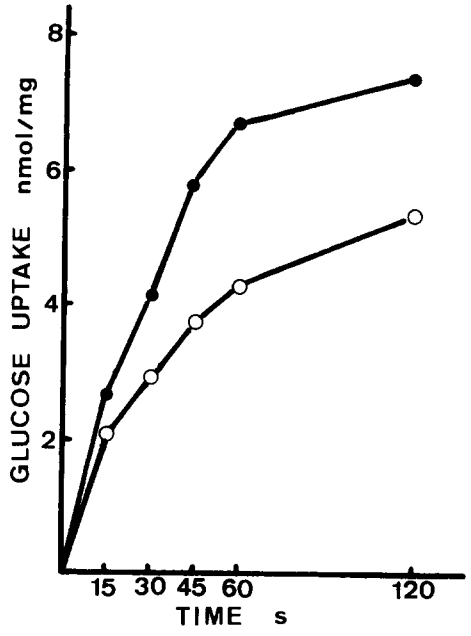

Fig. 6. Early time course of 0.2 mM D-[U-^{14}C]glucose uptake by intestinal brush-border membrane vesicles of *D. labrax* in the presence or in the absence of a Cl^- gradient: in > out. Vesicles were preloaded with 100 mM KCl at pH 7.4 and incubated in 50 mM Na_2SO_4 (●) (Cl^- gradient) or 100 mM NaCl (○) (no Cl^- gradient). Each point represents three determinations.

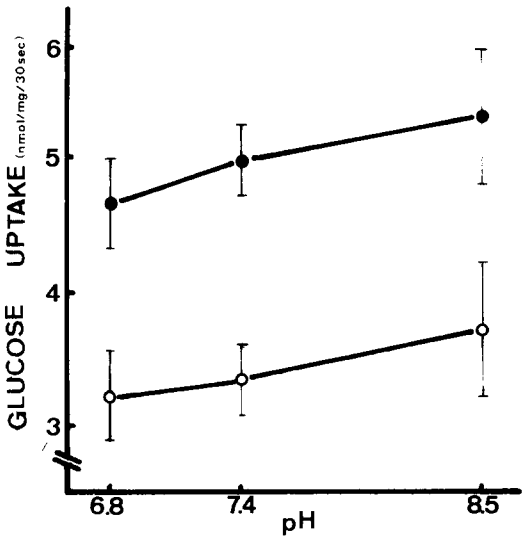

Fig. 7. Effect of pH on 0.2 mM D-[U-^{14}C]glucose transport by intestinal brush-border membrane vesicles of *D. labrax* in the presence or absence of a Cl^- gradient: in > out. Vesicles were preloaded with 100 mM KCl at the pH of incubation medium which contains Na_2SO_4 (●) (Cl^- gradient) or NaCl (○) (no Cl^- gradient). Values are means of four determinations. Each experiment is carried out at pH 6.5, 7.4 and 8.4 with Na_2SO_4 and NaCl.

TABLE V

INTRAVESICULAR AND EXTRAVESICULAR Cl^- EFFECTS ON 0.2 mM D-[U-^{14}C]GLUCOSE, 0.2 mM 2-AMINO[1-^{14}C]ISOBUTYRIC ACID OR 0.2 mM [U-^{14}C]GLYCINE UPTAKE IN THE ABSENCE OF AN Na^+ GRADIENT

Vesicles were preloaded with 100 mM NaCl and incubated in the presence of 50 mM Na_2SO_4 or 100 mM NaCl. Uptake was measured after 30 s at 25°C. The values are averages ± S.E. of four assays.

	Vesicles incubated in	
	Na_2SO_4	NaCl
Glucose uptake (nmol/mg protein per 30 s)	1.190 ± 0.318	0.612 ± 0.187
2-Aminoisobutyric acid uptake (nmol/mg protein per 30 s)	1.046 ± 0.091	0.936 ± 0.080
Glycine uptake (nmol/mg protein per 30 s)	1.293 ± 0.189	0.980 ± 0.139

or KCl in the incubation medium, glucose uptake cannot be stimulated by internal Cl^- (Table IV). Although Na^+ is necessary, the effect of Cl^- does not depend on a gradient of Na^+. Indeed, glucose uptake by vesicles preloaded with NaCl can still be stimulated when NaCl is replaced by Na_2SO_4 in the incubation medium. It is also the case for glycine transport (Table V) and to a less extent for 2-aminoisobutyric acid uptake.

Discussion

In the intestine of *D. labrax*, a double action of the chloride anion on the Na^+-dependent solute transport has been characterized. On the external side of the vesicles, Cl^- is required for amino acid transport, whereas on the internal side it stimulates glucose and amino acid uptake.

The external Cl^- dependence of amino acid transport in *D. labrax* confirms the presence, in the intestine of sea fish, of an original transport mechanism which was described for the first time in *B. salpa*, an herbivorous sea fish [6]. Probable

extension of its participation to amino acid transport in other fish can be presumed, since the two species studied up to now (*Boops* and *Dicentrarchus*) were chosen for availability and not on transport criteria.

In common with *B. salpa,* the Cl^--dependent amino acid transport of *D. labrax* is electrogenic, since it can be stimulated by a negative electrical potential (Table II). This result is consistent with previous observations showing, for intestinal preparations, that mucosal addition of amino acid (or glucose) evokes an increase in transepithelial potential difference [15,16].

The principal difference with *B. salpa* concerns a possible participation of a Cl^--insensitive mechanism to the Na^+-dependent amino acid uptake. In the absence of Cl^- (replaced by SO_4^{2-}) a residual Na^+-dependent transport has been characterized with glycine as with 2-aminoisobutyric acid. As is the case for the Cl^--sensitive component, this Cl^--insensitive component is electrogenic, since it can be stimulated by a negative inside potential (Table II). In *B. salpa,* this Cl^--insensitive component of amino acid transport is not entirely absent because it was observed that glycine (but not 2-aminoisobutyric acid) transport remains increased by Na^+ or by an electrical potential when Cl^- is replaced by SO_4^{2-} [6]. Nevertheless, its contribution to Na^+ amino acid transport seems less important than with *D. labrax.*

Although the external effect of Cl^- has not been characterized up to now in mammal intestine, it has been often described in membranes of synaptosomes, erythrocytes or platelets where it is concerned with the transport of γ-amino-*n*-butyric acid [17], proline [18], glycine [19–21] or aspartate [22]. The capacity of Cl^- to stimulate these transports has been interpreted as an effect of membrane potential due to a rapid diffusion of Cl^- [17]. In *D. labrax,* the effect of Cl^- cannot be restricted to an increase of negative charges inside the vesicles, since SCN^- would be more efficient, as it is for glucose transport, and this is not the case. Another explanation is that Cl^- may facilitate the fixation of the amino acid to the carrier [21]. The fact that Cl^- enhances the affinity of the transport of low 2-aminoisobutyric acid concentrations [Fig. 5] may support this hypothesis. However, in fish, the Cl^- dependence seems to result above all in an increase in V_{max} and in an effect on the non-saturable component of 2-aminoisobutyric acid uptake that suggest a more complex interaction with the carrier. In the case of human erythrocytes, arguments have been advanced suggesting cotransport of Cl^- and serotonine, and possible participation of Cl^- gradient in the energization of serotonine uptake [23]. In fish, the Cl^- gradient does not seem essential for 2-aminoisobutyric acid transport, since the magnitude of this uptake is not reduced when Cl^- concentrations inside and outside the vesicles are equilibrated (Table III). Moreover, we observed with intestinal vesicles of *D. labrax* incubated in NaCl and preloaded with Na_2SO_4 or NaCl, that in the absence of Na^+ gradient, a Cl^- gradient is not able to stimulate amino acid transport. The understanding of the role of Cl^- in fish could make use of the fact that the contribution of Cl^- to amino acid transport is reduced when the pH increases from 6.5 to 8.4 (Fig. 4), which suggests possible interactions between the anion and hypothetical electrical charges of the carrier or of the transported amino acid.

The effect of internal Cl^- is highly distinct from the effect of external Cl^-. It is nonspecific, because it concerns not only amino acid (although more glycine than 2-aminoisobutyric acid) but also, and more importantly, glucose transport (Tables III and IV). As was done for the external Cl^- requirement, we examined the possible distribution of this Cl^- internal effect with fish species. Stimulation of glucose transport can be obtained with vesicles of *B. salpa* incubated in Na_2SO_4 and preloaded in the presence of KCl instead of K_2SO_4 ($K_2SO_4 = 1.068 \pm 0.145$ nmol/mg protein per 30 s, KCl = 1.414 ± 0.153 nmol/mg protein per 30 s). This stimulation is, however, much lower in intensity than in *D. labrax* and is apparently not related to Cl^- gradient as in sea bass, because glucose transport by vesicles preloaded with KCl is not changed when Na_2SO_4 in the incubation medium is replaced by NaCl ($Na_2SO_4 = 1.414 \pm 0.153$ nmol/mg protein per 30 s, NaCl = 1.535 ± 0.131 nmol/mg protein per 30 s).

In *D. labrax,* the origin of the stimulating effect of internal Cl^- may result in the prolongation of the duration of the Na^+ and Cl^- transport. This assumption is based upon the observations that

this Cl^- effect is increased under a Cl^- gradient (in > out), that it requires the presence of Na^+ (Tables III and IV) and that it is attenuated during the early period (Fig. 6). Interactions between Na^+ and Cl^- transport have often been reported in mammals [24] as in fish [25], although recently the presumed mechanism has been questioned [26]. These interactions would explain how it is possible to stimulate glucose and amino acid transport by a Cl^--inverse gradient (in > out) under conditions of Na^+ equilibration (Tables III and IV). Conversely, a decrease in such transport may be expected in the absence of a Na^+ gradient but with high external Cl^- levels. This has been observed with *D. labrax* on vesicles preloaded with Na_2SO_4 when Na_2SO_4 in the incubation medium is replaced by NaCl (glucose uptake with $Na_2SO_4 = 0.365 \pm 0.072$ nmol/mg protein per 30 s, glucose uptake with $NaCl = 0.238 \pm 0.036$ nmol/mg protein per 30 s). Although the internal Cl^- requirement is of some interest for transport by sea fish brush-border membrane vesicles, it plays probably a more restricted physiological role because, in sea fish, the Cl^- concentration inside the enterocytes is surely much lower than in the sea. Per contra, the external Cl^- requirement may be of more physiological importance, because it occurs even with low concentrations and because it seems less influenced by concentration gradients.

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