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Fisiologia. — *Sodium-dependent amino acid transport in plasma membrane vesicles from rat liver* (*). Nota di MARIA GIOVANNA LEONARDI, ROBERTO COMOLLI e BARBARA GIORDANA, presentata (**) dal Corrisp. V. CAPRARO.

RIASSUNTO. — Sono state preparate vescicole di membrana plasmatica da epatociti di ratto secondo due differenti metodiche, per saggiarne l'idoneità come substrato per lo studio del trasporto degli aminoacidi, tappa fondamentale del metabolismo aminoacidico nel fegato. A tale scopo sono stati studiati i sistemi di trasporto dell'alanina e dell'istidina. Si è potuto così identificare una preparazione in grado di conservare la capacità di accumulare entrambi gli aminoacidi con modalità e specificità, sia riguardo al substrato che agli ioni cotrasportati, analoghe a quelle già osservate nell'epatocita isolato.

INTRODUCTION

Amino acid metabolism is one of the many functions of the liver. The hepatocytes convert the amino acids into a wide range of compounds, most of which will be released into the haematic circulation. Therefore the transport of amino acids across the plasma membrane of the hepatocyte is an essential event which may represent the rate limiting step in their metabolism, as it has been shown for alanine [1]. Transport experiments with purified plasma membrane vesicles represent a recent and successful improvement in the study of transport mechanisms, since it makes it possible to separate the transport phenomenon from the metabolic event, and it allows a definite control of the composition of the internal compartment.

In the present work, two different procedures for the preparation of plasma membrane vesicles from rat liver (LPV) were compared and some characteristics of L-alanine and L-histidine transport studied.

MATERIALS AND METHODS

Male Wistar rats (150 to 200 g), maintained in a light-controlled room (lights on from 7.00 to 19.00) at a temperature of 23 $\pm$ 1 °C, were used. The

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animals had free access to water and were fed ad libitum prior to use. The animals were killed by decapitation, the liver excised and rinsed in Krebs-Henseleit solution at 4 °C. The plasma membrane vesicles were then prepared either according to the method of Van Amelsvoort *et al.*, 1978 [2] as modified by Kraus-Friedman *et al.*, 1982 [3] or according to Epping and Bygrave, 1984 [4] with the following minor modifications: the crude homogenate was centrifuged at 1000 × *g* for 10 min and fraction A_3 was diluted 1 : 10 with 250 mM sucrose, 5 mM N-2-hydroxyethylpiperazine-N'2-ethansulfonic acid (HEPES)-KOH pH 7.5 and centrifuged at 100000 × g for 1 h, in order to obtain the separation between the plasma membranes and the Percoll glassy pellet.

The purity of the vesicle preparation was tested assaying the activity of marker enzymes in the crude homogenate and in the final pellet: Na-K ATPase was assayed according to Schoner *et al.*, 1967 [5]; 5'-nucleotidase according to Michell and Hawthone, 1965 [6]; cytochrome *c* oxidase according to Smith, 1955 [7] and NADPH cytochrome *c* reductase according to Masters *et al.*, 1967 [8]. Alkaline phosphatase was assayed with a Boehringer kit (Nr. 415278) with *p*-nitrophenylphosphate as a substrate. Proteins were determined by the method of Bradford, 1976 [9], with a Bio-Rad kit, using bovine serum albumin as standard. The transport experiments were performed in triplicate or quadruplicate at room temperature mixing an appropriate aliquot of the vesicle suspension (at a final protein concentration of 5-10 mg/ml) to an incubation mixture containing 250 mM sucrose, 0.4 mM $CaCl_2$, 20 mM $MgCl_2$, 15 mM HEPES-KOH pH 7.5, L-^{14}C-alanine and L-^{3}H-histidine (purchased from Amersham International plc. UK, sp. act. 150 mCi/mmol and 40 Ci/mmol respectively) and salt gradients were added at the concentrations indicated in the legends of the tables and the figures. At selected times, 20 μl samples were withdrawn from the incubation mixture, diluted with 2 ml of ice-cold stop solution (250 mM sucrose, 0.2 mM $CaCl_2$, 100 mM NaCl, 10 mM HEPES-KOH pH 7.5), filtered through a 0.45 μm pore size cellulose-nitrate filter (MFS Micro Filtration System) and rapidly washed with 3 ml of ice-cold stop solution. The filters were then dissolved in a scintillation mixture and the radioactivity counted in a liquid scintillation spectrometer (Packard, Model 300 C). Short time incubations (1-10 s) were performed in quadruplicate with an automated device assembled in our laboratory consisting of a timer which controls both a shaker and an injector (Automatic Dispenser, Oxford, Athy, Ireland). The experimental procedure was similar to that reported by Kessler and Semenza (1983) [10].

Results and discussion

The LPV preparations obtained with the two methods were assayed for purity by means of specific marker enzymes (Table I). Both final pellets appear to be enriched to the same extent, with respect to the initial homogenate, as far as the plasma membrane marker enzyme Na-K ATPase is concerned.

However, 5'-nucleotidase enrichment factor is lower and alkaline phosphatase is higher than that reported in the original papers (2, 4). In contrast, the two preparations differ strikingly with respect to microsomal contamination, tested as NADPH cytochrome *c* reductase activity. Following the procedure of Epping and Bygrave [4], LPV almost free from major cellular contaminants were obtained (Table I).

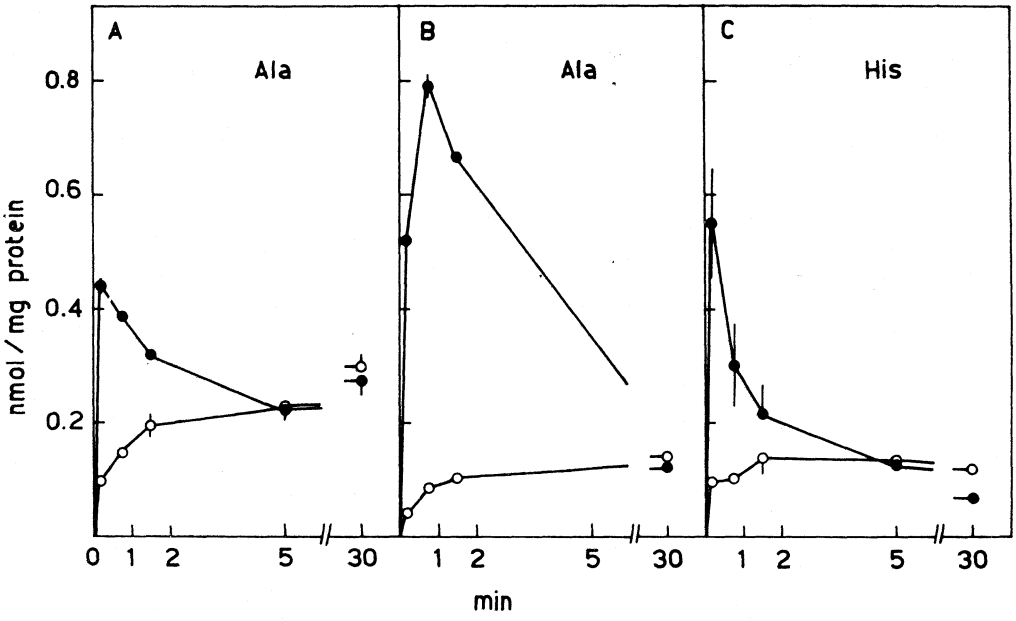

Fig. 1. - Time course of alanine and histidine in rat liver plasma membrane vesicles prepared according to Van Amelsvoort *et al.*, 1978 (A) and Epping and Bygrave, 1984 (B-C) - LPV, re-suspended in 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, were incubated in a medium of the following final composition: 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 100 mM NaSCN (●) or 100 mM KSCN (○) and 0.2 mM L-^{14}C-alanine (A-B) or 0.2 mM L-^{3}H-histidine (C). Each point represents the mean ± S.E. of a typical experiment carried out in triplicate. When not given S.E. bars were smaller than the symbols used.

A typical time course of alanine uptake into the vesicles, measured in the two preparations, in the presence of an inwardly directed sodium or potassium gradient, is reported in fig. 1 (A, B). A transient intravesicular accumulation of the amino acid is present in both preparations, but its absolute value, calculated as the ratio between the maximal (at 45 s) and the equilibrium (at 40 min) uptake values, differred noticeably. The accumulation ratio was 1.6 ± 0.1 (Mean ± SE, 16 experiments) in LPV following Sips *et al.*, 1980 [11] (fig. 1A) and 5.4 ± .4 (Mean ± SE, 6 experiments) in LPV following Epping and Bygrave, 1984 [4] (fig. 1 B). In the first case, the value is lower than that reported by the authors, but it is in good agreement with that found by Samson and Fehlmann, 1982 [12], in LPV from fresh isolated hepatocytes prepared by the same method. In the latter case, a much higher accumulation ratio is obtained,

TABLE I.

Enzyme activities measured in the homogenate and plasma membrane vesicles (*).

	A			B		
	H	LPV	R.E.	H	LPV	R.E.
Na^+-K^+ ATPase	28.6 ± 8.5 (11)	88.3 ± 11.5 (11)	5.2 ± 1.4 (11)	39.4 ± 8.3 (7)	339.7 ± 95.6 (7)	8.2 ± 1.6 (7)
5'Nucleotidase	17.6 ± 7.3 (11)	60.5 ± 14.2 (11)	4.7 ± 0.9 (11)	—	—	—
Alkaline phosphatase	—	—	—	4.7 ± 0.5 (3)	106.2 ± 18.4 (3)	25.5 ± 6.8 (3)
NADPH Cyt. c reductase	22.3 ± 10.7 (10)	79.9 ± 29.4 (10)	4.9 ± 1.1 (10)	25.3 ± 4.9 (5)	8.2 ± 0.9 (5)	0.4 ± 0.1 (5)
Cytochrome c oxidase	13.4 ± 6.6 (11)	1.6 ± 0,4 (11)	0.2 ± 0.05 (11)	5.9 ± 1.1 (5)	1.8 ± 0.9 (5)	.25 ± 0.1 (5)

(*) LPV were prepared according to Van Amelsvoort *et al.*, 1978 [2] (A) and according to Epping and Bygrave, 1984 [4] (B). Activities are expressed as nmoles min^{-1} mg $protein^{-1}$, Cytochrome *c* oxidase is espressed as a first order rate constant (min^{-1} mg $protein^{-1}$). H-homogenate, LPV-plasma membrane vesicles, R.E.-relative enrichement values obtained by dividing plasma membrane specific activities by specific activities of homogenate. Numbers in brackets are number of experiments.

which could be due to different reasons (see Heinz and Weinstein, 1984 [13]), but it could primarily be ascribed to the higher degree of purification of the plasma membranes. The lack of the microsomal contamination eliminates the presence of a vesicular space accessible to alanine by means of an Na-independent uptake, as indicated by the higher alanine uptake in the absence of Na in 1 A than in 1 B. It should be remembered that the hepatocytes are able to accumulate alanine extensively, the intracellular amino acid concentration reaching in 20 min a value 22-fold higher than the extracellular one [14].

On the basis of these experiments, alanine and histidine uptakes were further studied in the latter LPV preparation, in order to verify whether this vesicle preparation retains the characteristics which have been assessed in isolated hepatocytes. The amino acids chosen were alanine, which is known to be transported by two transport agencies, system A and system ASC [15], and histidine, which can be considered a selective substrate for system N [15, 16]. Fig. 1 C shows that histidine is also accumulated within the vesicles, with an accumulation ratio of 7.8 $\pm$ 1.8 (Mean $\pm$ SE, 4 experiments) in the presence of an inwardly directed sodium gradient. The Na-independent histidine uptake at 10 s is 19 $\pm$ 4% (Mean $\pm$ S.E., 4 experiments) of the total uptake in the presence of sodium.

TABLE II.

Effect of monovalent cations on alanine and histidine uptakes (*).

Salt present	Alanine %	Histidine %
LiCl	68	55
NaCl	100	100
KCl	7	32
RbCl	9	29
CsCl	7	27
CholineCl	—	39

(*) LPV, resuspended in 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, were incubated in medium of the following final composition: 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 100 mM of indicated salts. The uptakes were terminated after 40 s for alanine and 8 s for histidine. The uptake values are expressed as a percentage of the value obtained in the presence of NaCl gradient (see text).

The cation specificity of the transport agencies involved in the two amino acid uptakes was tested at 40 s and 8 s incubation for alanine and histidine respectively (Table II). The effects of cations are expressed as % of the value obtained in the presence of the NaCl gradient (1.06 $\pm$ 0.02 nmol mg protein^{-1}, Mean $\pm$ S.E., 3 experiments for alanine and 0.20 $\pm$ 0.06 nmol mg protein^{-1},

Mean ± S.E., 3 experiments for histidine). Lithium can efficiently substitute for sodium in catalyzing alanine transport across LPV; initially lithium was supposed to be accepted by system ASC only in rat hepatocyte [17], but subsequently a small lithium supported alanine uptake mediated by system A has been demonstrated by Kilberg *et al.*, 1981 [18]. Lithium can also support histidine uptake through system N, as described by Kilberg *et al.*, 1980 [16].

TABLE III.

Effect of anions on alanine and histidine uptakes (*).

Salt present	Alanine %	Histidine %
NaSCN	100	100
NaCl	85	100
Na_2SO_4	52	105
NaGluconate	43	93

(*) LPV, resuspended in 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 100 mM of the indicated salts; Na_2SO_4 50 mM. The uptakes were terminated after 40 s for alanine and 8 s for histidine. The uptake values are expressed as per cent of the value obtained in the presence of the NaSCN gradient (see text).

The electrogenicity of the systems involved in the transport of the two amino acids was also studied. For this purpose, alanine and histidine uptakes were measured in the presence of an inwardly directed sodium gradient obtained with sodium salts, whose anions have a different permeability across the plasma membrane: $P_{SCN} > P_{Cl} > P_{SO4} > P_{gluconate}$. The thiocyanate anion, having a higher permeability than sodium, generates a transmembrane electrical potential difference with the negative pole inside the vesicle: the absolute value of the potential generated by the different anions progressively decreases, and eventually inverts its polarity according to the anion permeabilities, gluconate being impermeable. In Table III the uptake values are expressed as per cent of the values obtained with NaSCN (1.25 ± 0.04 nmol mg protein^{-1}, Mean ± ± S.E., 3 experiments for alanine, and 0.19 ± 0.05 nmol mg protein^{-1}, Mean ± ± S.E., 3 experiments for histidine). Alanine transport is dependent on the transmembrane potential since a decreasing rate of uptake of the amino acid takes place according to the permeabilities of the sodium counterions whereas histidine uptake is unaffected. The electrogenicity of alanine transport had already been assessed in isolated rat hepatocytes [14], and was confirmed by the data obtained by Sips *et al.*, 1980 [11] as well as Quinlan *et al.*, 1982 [19] in their vesicle preparation. To our knowledge, the electroneutral nature of histidine uptake has been established here for the first time.

Alanine transport occurs via the two transport systems A and ASC; the alanine component which cross the membrane through the A system can be traced with inhibition experiments in the presence of α-(methylamino) isobutyric acid (MeAIB), model substrate for system A. The effect of a 50-fold excess concentration of MeAIB was therefore tested on the uptake of alanine 0.2 mM at 5 s in order to avoid aspecific inhibition due to the dissipation of the sodium gradient. The rate of the Na-dependent uptake of the labelled amino acid in the absence of the inhibitor was 0.32 ± 0.09 nmol mg protein^{-1}

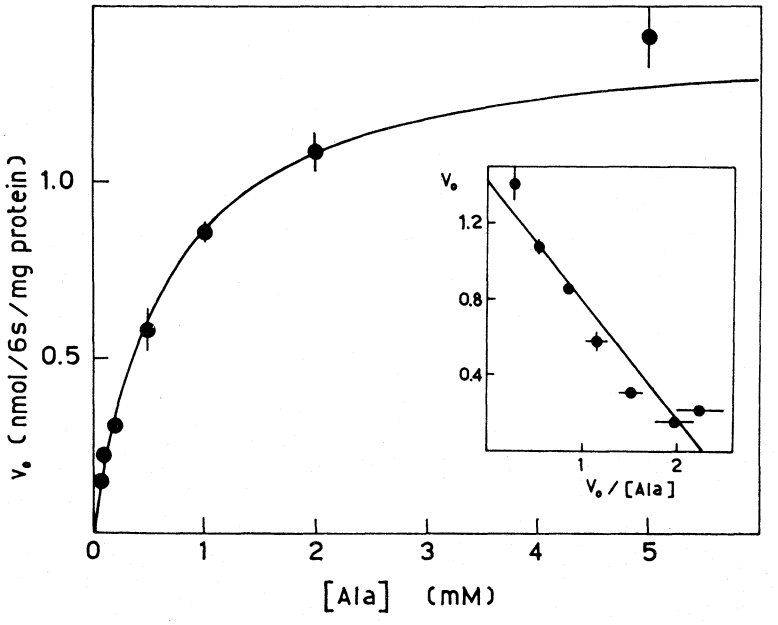

Fig. 2. - Kinetics of alanine uptake - LPV, re-suspended in 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, were incubated in a medium of the following final composition: 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 100 mM NaSCN and 0.075 to 5 mM L-^{14} C-alanine. Uptake was measured after 6 s incubation. The inset shows the Eadie-Hofstee plot of the data. Each point represents the mean ± S.E. of a typical experiment carried out in quintuplicate. When not given, S.E. bars were smaller than the symbols used.

(Mean ± S.E., 3 experiments) and 0.10 ± 0.02 nmol mg protein^{-1} (Mean ± ± SE, 3 experiments) with MeAIB, i.e. MeAIB causes a 70% inhibition of the sodium sensitive uptake of alanine, which confirms that this amino acid is taken up by the hepatocyte mainly through the A system. This result is in keeping with previous data obtained in isolated cells [20, 21]. The ASC component could not be determined since a 25-fold excess concentration of cysteine, model substrate for system ASC, caused in the same experiments, a 100% inhibition of alanine uptake: besides, cysteine is known to be a non-competitive inhibitor of the A system [18].

The initial uptake rates of alanine and histidine as a function of substrate concentrations (between 0 and 5 mM) were also studied. In order to obtain accurate values of K_m and V_{max}, short time uptakes (between 2 and 10 s) were measured at 0.2 and 5 mM concentrations (data not shown). The uptakes with time of both alanine and histidine were linear up to 10 s. Therefore uptakes at 6 s for alanine and 8 s for histidine were measured to obtain the kinetic constants. From the graphs of the initial uptake rates it can also be evinced that no binding to LPV of alanine takes place, whereas a small but definite (about

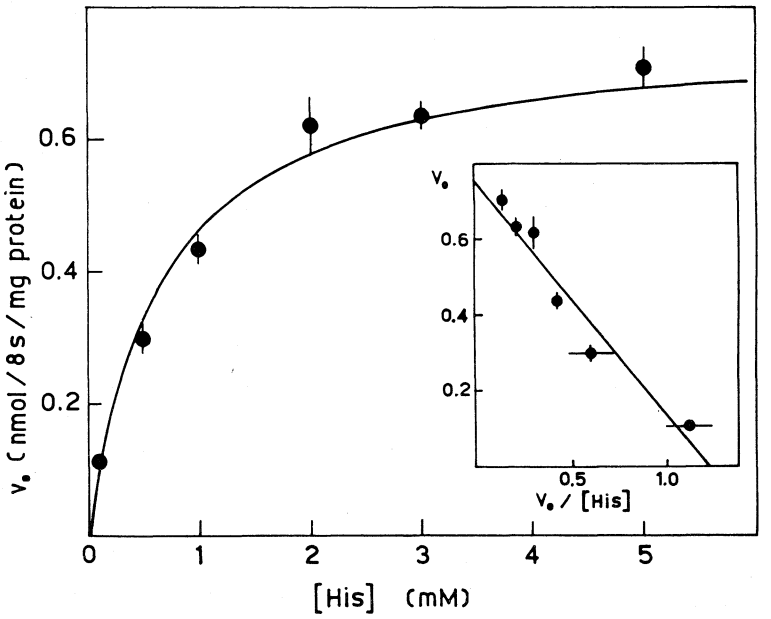

Fig. 3. - Kinetics of histidine uptake - LPV, re-suspended in 250 mM sucrose, 5 mM HEPES-KOH pH 7.5, were incubated in a medium of the following final composition: 250 mM sucrose, 6 mM HEPES-KOH pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 100 mM NaSCN and 0.1 to 5 mM L-^{3}H-histidine. Uptake was measured after 8 s incubation. The inset shows the Eadie-Hofstee plot of the data. Each point represents the mean ± S.E. of a typical experiment carried out in quintuplicate. When not given, S.E. bars were smaller than the symbols used.

11% of total uptake at 8 s) binding of histidine to the vesicles occurs at short time incubations, as shown by the non-zero intercept on the y-axis. Fig. 2 and fig. 3 report the kinetics of alanine and histidine, respectively, in the presence of a NaSCN gradient; in both cases a saturation kinetics is obtained and no appreciable linear component is evident. On the contrary, a considerable linear component was present in the alanine kinetics measured by Sips *et al.*, 1980 [11], presumably due to the microsomal contamination in their preparation. The Eadie-Hofstee plots of the data, reported in the inset of each figure, yield straight lines, from which the kinetic parameters K_m and V_{max} were calculated.

For alanine the constants were 0.63 ± 0.08 mM and 1.42 ± 0.12 nmol $6\ s^{-1}$ mg protein^{-1} respectively. The K_m value is in good agreement with the value obtained by Fehlman *et al.*, 1979 [22] and Dorio *et al.*, 1984 [23] in isolated hepatocytes for the high affinity component of the transport measured for α-aminoisobutyric acid (AIB) and MeAIB; the K_m value, however, is considerably lower than that obtained by Sips *et al.*, 1980 [11]. For histidine the K_m values is 0.50 ± 0.06 mM and V_{max} is 0.63 ± 0.04 nmol $8\ s^{-1}$ mg protein^{-1}. No data have been found on the kinetics of histidine in literature. Histidine permeates through the N system which is shared by glutamine: for this amino acid a K_m value of 1.1 mM has been found in isolated hepatocytes [16].

From the data reported in this paper, it can be concluded that LPV prepared following Epping and Bygrave, retain the transport properties observed in the intact cells from which the membranes were obtained. In fact the characteristics found for the different transport agencies, as well as the specificities of the carriers for both substrate and co-ions are in reasonable agreement with those found in intact hepatocytes.

Acknowledgements.

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