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Fisiologia. — *Alanine transport in purified plasma membrane vesicles from ascites hepatoma (Yoshida AH-130) cells in stationary phase of the in vivo growth* (*). Nota di MARIA GIOVANNA LEONARDI, ELENA CISLAGHI, PATRIZIA BONFANTI, ROBERTO COMOLLI e BARBARA GIORDANA, presentata (**) dal Socio V. CAPRARO.

ABSTRACT. — An increased amino acid uptake occurs in neoplastic cells. In order to discriminate between cell metabolic effects and/or membrane changes, the transport of L-alanine has been studied in the present work using plasma membrane vesicles isolated from a highly undifferentiated ascites hepatoma cell line (Yoshida AH 130).

Contrary to the data previously obtained with normal hepatocytes, in which alanine transport occurs via two different transport systems (A and ASC), kinetic studies with hepatoma membrane vesicles showed the presence of a unique component.

However, the presence of two transport systems cannot be excluded in this hepatoma cell line, since the technique employed can only barely detect transport systems with similar kinetic constants.

KEY WORDS: Plasma membrane vesicles; Alanine transport; Kinetic parameters; Ascites hepatoma.

RIASSUNTO. — *Trasporto di alanina in vescicole di membrana plasmatica purificate da cellule di epatoma ascite di Yoshida (AH 130) in fase stazionaria di crescita.* È stata studiata l'incorporazione di L-alanina in vescicole di membrane plasmatiche isolate da cellule, in fase stazionaria di crescita di un tumore sperimentale altamente indifferenziato, come l'epatoma di Yoshida AH-130. L'utilizzazione, nello studio dei meccanismi di trasporto, di frammenti vescicolati di membrana, invece delle cellule, permette di escludere le eventuali interferenze metaboliche; ciò è particolarmente utile nelle cellule trasformate, in cui si ha un incremento nell'assunzione di diversi substrati che potrebbe dipendere o da alterazioni primarie dei sistemi di trasporto o dall'aumento del metabolismo.

Misurando l'incorporazione nel tempo della L-alanina nelle vescicole è stato evidenziato un differente andamento rispetto a quanto osservato in vescicole di membrana plasmatica da fegato di ratto. Lo studio della cinetica mostra, contrariamente a quanto osservato negli epatociti in cui l'alanina entra attraverso due sistemi di trasporto (A e ASC), la presenza di un solo trasportatore. L'evidenziazione di una sola componente a saturazione potrebbe, però, dipendere dall'impossibilità di discriminare in base ai soli esperimenti cinetici, due sistemi con costanti cinetiche poco diverse.

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Introduction

It has been previously demonstrated that the neoplastic transformation increases the uptake of certain sugars (1-3) and amino acids (4-6) in cultured animal cells. This alteration of nutrient uptake could be the consequence of primary changes in the rate of cellular metabolism or of membrane transport. Actually, the plasma membrane modifications of tumor cells have been considered as one of the main aspects of neoplastic transformation (7).

It appeared of interest, therefore, to investigate the possible changes of membrane amino acid transport in tumor cells using a purified plasma membrane vesicle preparation isolated from a highly undifferentiated ascites hepatoma (Yoshida AH130). Transport experiments performed with vesiculated plasma membrane fragments make it possible to separate the transport phenomena from the metabolic events.

Materials and methods

L-(U-^{14}C)-Alanine 150 mCi/mmol was purchased from Amersham International (Amersham, UK). Cytochrome C, Type VI, was purchased from Sigma (St. Louis, USA). All other reagents were analytical grade products from BDH (Italy).

Yoshida (AH-130) ascites hepatoma cells were propagated by injection (50-60 $\times$ 10^6 cells) into the peritoneal cavity of male Wistar rats, maintained in a light controlled room (light on from 7.00 to 19.00) at a temperature of 23 $\pm$ 1°C. Stationary phase ascites cells were harvested on day 12 after inoculation (8). The cells were washed to remove the ascitic fluid and contaminating erythrocytes with a medium of the following composition: 146 mM NaCl, 35 mM Tris-HCl buffer pH 7.6 (medium A). The cells were counted and then stored in medium A plus 10% glycerol at - 80°C in 2.5 ml aliquots containing 100 $\times$ 10^6 cells/ml.

For the preparation of the plasma membrane vesicles (PMV), the cells were thawed rapidly at 37°C, centrifuged and resuspended in 10 ml of a medium of the following composition: 250 mM sucrose, 2 mM N-2-hydroxyethylpiperazine-N'-2-ethan-sulfonic acid (HEPES)-Tris buffer pH 7.5 (medium B), added with 0.1 mM phenylmethylsulfonylfluoride (PMSF). PMV were prepared according to Boumendil-Podevin and Podevin (9), with the following modifications: 5.8 $\times$ 10^8 cells/5 ml were homogenized and the crude homogenate was diluted 1:2 v/v with medium B. After centrifugation at 1000 $\times$ g for 10 min., the supernatant was saved and the pellet (P_1) was resuspended in medium B and recentrifuged. The combined supernatants (S_1 + S_2) were centrifuged at 22000 $\times$ g for 15 min.; the supernatant (S_3) was discarded and the upper fluffy layer of the pellet was fractionated on the self-generating Percoll gradient. The centrifugation, at 40000 $\times$ g for 15 min, gave only two major bands. The upper band (F_1), containing the plasma membrane fraction,

was resuspended in medium B and centrifuged at 100000 × g for 1 h, in order to obtain the separation between the plasma membranes and the Percoll glassy pellet. The overall yield of the procedure was 1.2 ± 0.19% of homogenate total protein (Mean ± S.E., 9 determinations). The addition of 1 mg/ml DNAse to the combined supernatants greatly improved the final pellet, which was otherwise rather viscous.

The purity of the vesicle preparation was tested by assaying the specific activity, in the crude homogenate and in the final pellet, of two marker enzymes of the plasma membrane: the Na^+-K^+ ATPase determined according to Schoner *et al.*, 1967 (10) and the 5'nucleotidase, assayed according to Michell and Hawthone, 1965 (11). The presence of contaminating membranes from the microsomal fraction and from mitochondria was determined, respectively, by assaying the NADPH cytochrome c reductase activity according to Masters *et al.*, (12) and the cytochrome c oxidase, according to Smith (13). Protein was determined by the method of Bradford (14), with a Bio-Rad Kit, using bovine serum albumin as standard.

The transport experiments were performed at 25°C by a rapid filtration technique as reported in a previous paper (15).

Results and discussion

The characterization of alanine transport has been performed in plasma membrane vesicles form Yoshida hepatoma cells obtained by a self-forming gradient of Percoll, according to Boumendil-Podevin and Podevin, 1983 (9). The vesicle suspension shows a satisfactory degree of purity, since the enrichment factor (ratio between the enzyme specific activity in the plasma membrane fraction and in the homogenate) of the two plasma membrane marker enzymes 5'nucleotidase and Na^+-K^+ ATPase, is higher than 14 (14.3 ± 3.9, Mean ± S.E., 4 preparations and 15.3 ± 4.5 Mean ± S.E., 5 preparations, respectively). The preparation is only slightly contaminated by mitochondria (enrichment factor for cytochrome c oxidase, 1.99 ± 0.73, Mean ± S.E., 5 preparations) and it is almost free from microsomes.

Alanine uptake with time has been measured in vesicle preparations obtained from cells stored at −80°C. The uptake values, measured in the presence of an inwardly directed sodium or potassium gradient, are reported in fig. 1: a transient intravesicular accumulation of the amino acid occurred only in the presence of the sodium gradient. The accumulation ratio, calculated as the ratio between the maximal and the equilibrium uptake values, was 2.4 ± 0.11 (Mean ± S.E., 4 preparations).

This value is strictly similar to that observed in vesicles isolated from Yoshida cells utilized on the same day of harvesting (accumulation ratio 2.3 ± 0.25 Mean ± S.E., 3 preparations).

The pattern of the time course in these preparations differs noticeably from that observed in liver plasma membrane vesicles. The time at which the maximal uptake

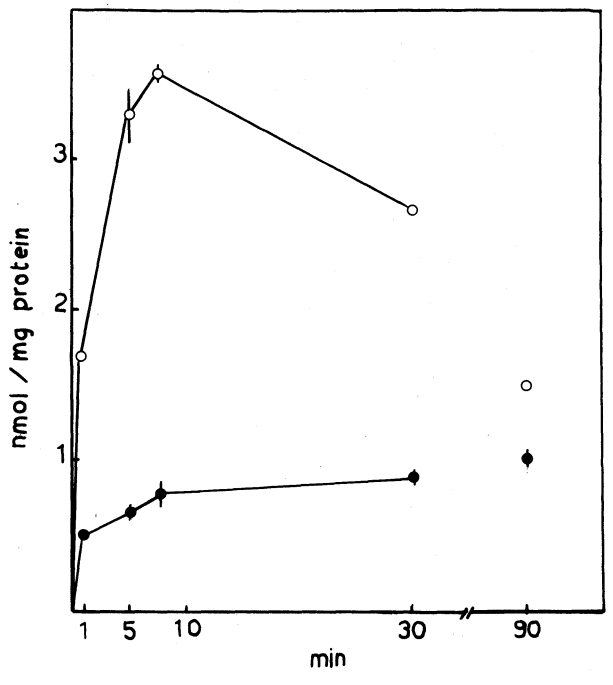

Fig. 1. - Time course of alanine uptake in plasma membrane vesicles from Yoshida hepatoma cells - Plasma membrane vesicles, resuspended in 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, were incubated in a medium of the following final composition: 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, 100 mM NaSCN (○) or 100 mM KSCN (●) and 0.2 mM L-^{14}C-alanine. 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.

value is reached, shifts from 45 s in liver plasma membrane vesicles (15-17) to 7 min and 30 s in Yoshida PMV.

The total intravesicular volume is 2.71 ± 0.48 μl/mg protein (Mean ± S.E., 9 preparations), as calculated from the equilibrium values.

The cation specificity of the transport system(s) of alanine was tested after 15 s of incubation (table 1). The maximal uptake value is observed in the presence of sodium; lithium could also support the amino acid uptake whereas potassium was unable to accelerate alanine entry into the vesicles, the uptake value being equal to that obtained in the presence of the impermeable cation choline$^+$. Lithium is accepted by system ASC in rat hepatocytes (18) and it has been recently shown that lithium can also support alanine uptake mediated by system A (19).

Table 2 shows that alanine translocation is electrogenic in Yoshida PMV. The amino acid uptake was measured, at 10 s and 7 min 30 s, in the presence of an inwardly directed sodium gradient, with sodium counterions with different permeability coefficients across the plasma membrane. If the thiocyanate anion has a permeability coefficient higher than sodium, it generates a trasmembrane electrical potential difference with the negative pole inside the vesicle. The value of the poten-

tial generated by the different anions progressively decreases and eventually inverts its polarity, according to the anion permeability. Alanine transport is dependent on the transmembrane potential since a decreasing rate of uptake of the amino acid takes place according to the presumptive permeabilities of the sodium counterions. The electrogenicity of alanine transport has been demonstrated both in isolated rat

Table 1. – *Effect of monovalent cations on alanine uptake* *.

Salt present	alanine uptake	
	pmoles/mg of protein	(%)
LiCl	340 ± 50	88
NaCl	390 ± 10	100
KCl	170 ± 20	43
CholineCl	150 ± 5	38

* Plasma membrane vesicles, resuspended in 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, were incubated in a medium of the following final composition: 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, 100 mM of the indicated salts. The uptakes were terminated after 15 s. Means ± S.E. of an experiment carried out in triplicate. The uptakes are also expressed as per cent of the value obtained in the presence of NaCl gradient.

Table 2. – *Effect of anions on alanine uptake* *.

Salt present	alanine uptake			
	A		B	
	pmoles/mg of protein	(%)	pmoles/mg of protein	%
NaSCN	520 ± 10	100	3276 ± 40	100
NaCl	445 ± 10	85	2744 ± 10	85
NaGluconate	367 ± 10	70	1568 ± 18	48

* Plasma membrane vesicles, resuspended in 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, were incubated in a medium of the following final composition: 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, 100 mM of the indicated salts. The uptakes were terminated after 10 s (A) or after 7 min 30 s (B). Means ± S.E. of an experiment carried out in triplicate. The uptakes are also expressed as per cent of the value obtained in the presence of NaCl gradient.

hepatocytes (20) and in liver plasma membrane vesicles (15,17,21).

The dependence of alanine transport on external Na concentration was measured in the presence of a constant transmembrane electrical potential difference generated by SCN^- diffusion (KSCN substituted for NaSCN). The uptake of alanine displays a saturation kinetics. An Na-independent component is also present,

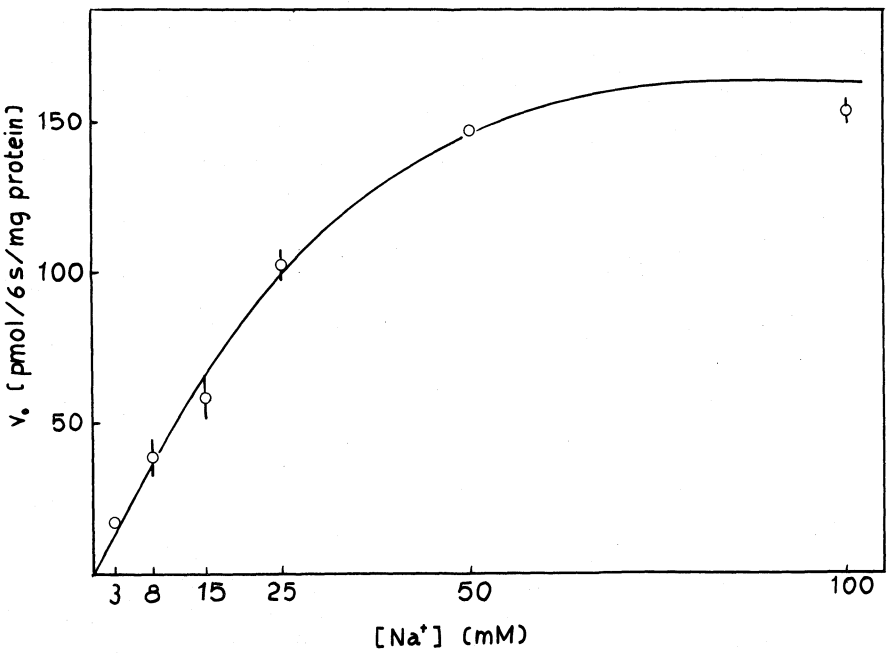

Fig. 2. – Kinetics of alanine uptake as a function of external sodium concentration – Plasma membrane vesicles, resuspended in 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, were incubated in a medium of the following final composition: 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, 0.2 mM L-^{14}C-alanine and 3 to 100 mM NaSCN substituted by an equal amount of KSCN. The uptakes were terminated after 6 s of incubation. 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.

as shown by the non-zero intercept on the ordinate. Fig. 2 reports the uptake values after substraction of the Na-independent component. Since the relationship is essentially hyperbolic, the Na : amino acid stoichiometry is 1 : 1.

In order to obtain accurate values of the transport kinetic parameters K_m and V_{max}, uptakes between 2 and 10 s were measured at 0.2 and 5 mM concentration in the presence of a NaSCN or a KSCN gradient (data not shown). Since the uptake with time of alanine was linear up to 10 s, the kinetic experiments were performed at 6 s of incubation. No aspecific binding of the amino acid to the vesicles is present at short-time incubations. Fig. 3 reports the kinetics of alanine as a function of external alanine concentration (between 50 μM and 5 mM) in the presence and in the absence of a Na^+ gradient (KSCN substituted for NaSCN). The kinetics of the amino acid is consistent with a saturable component plus a linear one. The diffusional constant (K_d) calculated from the straight part of the curve in the presence of Na^+ is equal to that calculated from the curve in the absence of Na^+, which shows no evidence of the presence of a saturable component. Therefore, alanine kinetics in the

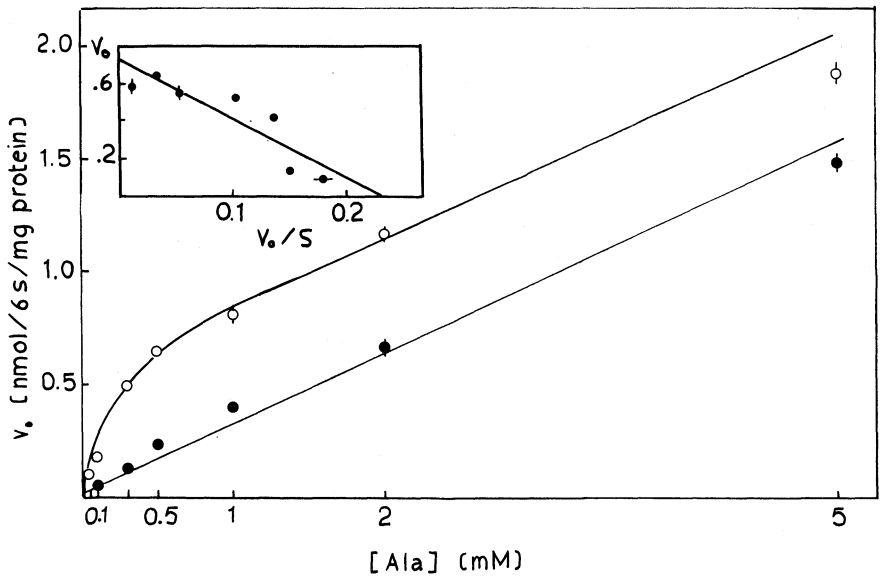

Fig. 3. – Kinetics of alanine uptake as a function of external amino concentration (0-5 mM) – Plasma membrane vesicles, resuspended in 250 mM sucrose, 2 mM HEPES-Tris pH 7.5, were incubated in a medium of the following final composition: 250 mM sucrose, 2mM HEPES-Tris pH 7.5, 100 mM NaSCN (o) or KSCN (•) and 0.05 to 5 mM L-^{14} C-alanine. The uptakes were terminated after 6 s of incubation. The inset shows the Eadie-Hofstee plot of the data corrected for the Na-independent component. 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.

absence of Na^+ is a good tracer for the computation of the diffusional component of the overall transport in the presence of the cation. The Eadie-Hofstee plot of the data corrected for the linear component (inset in fig. 3) yields a straight line consistent with the presence of one carrier system, with $K_m = 0.305 \pm 0.060$ mM and $V_{max} = 715 \pm 77$ pmole/6 s/mg protein.

Alanine transport occurs in hepatocytes (22) and HTC hepatoma cells (23) via the two transport systems A and ASC. To verify if both systems are also operative in Yoshida hepatoma cells, the initial uptake of alanine vs external alanine concentration was more accurately measured between 10 and 1000 μM concentration range (fig. 4). Alanine kinetics in the absence of Na^+ was also determined as a measure of the diffusional component. If alanine is transferred through two different transport pathways the Eadie-Hofstee plot of the data might show a curvilinear relationship.

Instead, the plot of the data corrected for the diffusional component (inset), is again consistent with the presence of only one carrier system with $K_m = 0.150 \pm 0.026$ mM and $V_{max} = 505 \pm 54$ pmole/6 s/mg protein. However, the

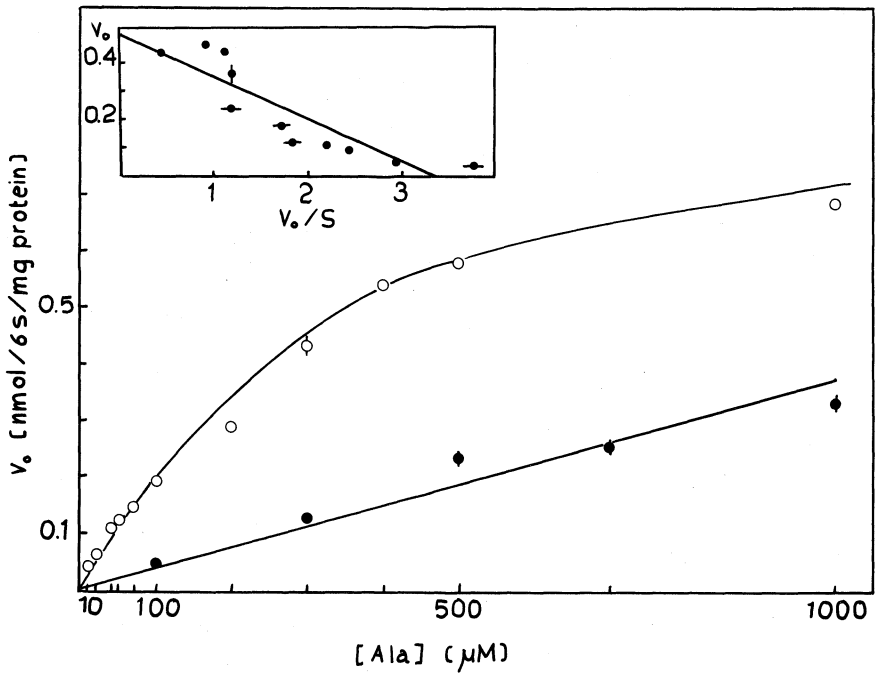

Fig. 4. - Kinetics of alanine uptakes as a function of external aminoacid concentration (0-1 mM) - Experimental conditions as decribed in fig. 3.

Eadie-Hofstee plot cannot resolve two components, if their kinetic constants do not differ greatly (24): therefore, the possible identification of two carrier systems, in Yoshida AH 130 cells in stationary phase, would be obtained only with inhibition experiments.

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