Biochimica et Biophysica Acta, 1026 (1990) 1–12
Elsevier

BBAMEM 74882

Na^+-dependent transport of alanine and serine by liver plasma-membrane vesicles from rats fed a low-protein or a high-protein diet

Geneviève Bourdel, Marguerite Forestier and Betty Gouhot

Centre de Recherche sur la Nutrition, C N R S, Meudon (France)

(Received 21 February 1990)

Key words Alanine, Serine, Sodium ion dependent transport, Membrane vesicle, High protein diet, (Rat liver)

Plasma-membrane vesicles prepared from the liver of rats fed either a low-(LP) or a high-protein (HP) diet exhibited Na^+-dependent active transport of alanine and serine. The process gave apparent kinetic parameters compatible with a single saturable component for both amino acids. Na,K-ATPase (EC 3.6.1.37), marker of the basolateral domain of the hepatocyte plasma-membrane, was chosen as reference for the expression of amino acid transport in vesicle preparations. The high-protein diet induced a significant increase in liver Na,K-ATPase activity also found in corresponding plasma-membrane preparations, in parallel with an increase in the capacity towards amino acid transport. This suggests that in rats fed the high protein diet, transcellular Na^+ exchange, although increased, remains well balanced. *N*-Methylaminoisobutyric acid (MeAIB), due to its poor velocity, proved unsuitable to distinguish between systems A and ASC in the experimental model. Comparing Na^+- and Li^+-driven transport, a family of carriers with strict Na^+-dependency (A-like) was evidenced in LP vesicles but not in HP vesicles. The sensitivity to the lowering of the pH from 7.5 to 6.5 in the external medium was similar in both type of vesicles when Na^+ was the driving ion. In the HP vesicles the Li^+-tolerant, pH-insensitive component (ASC-like) was increased in parallel with overall Na^+-dependent transport. These functional properties suggest that the carriers involved in the stimulation of transport in HP vesicles are composite in nature. Increasing concentrations of an amino acid mixture mimicking the changes of portal aminoacidemia inhibited the transport of alanine and of serine. The degree of inhibition was correlated with the relative concentration of substrate and was independent of the nutritional treatment.

Introduction

It has been recognized for a long time that amino acids are transported across the plasma-membrane of the hepatocyte by several carrier systems [1,2] Isolated hepatocytes have been a most useful model for the functional characterization of the two main Na-dependent carrier systems (A and ASC) involved in the transport of neutral amino acids. However, the capacity of the liver for amino acid catabolism is highly susceptible to adaptive regulation and can interfere through changes in intracellular amino acid concentration with tracer-determined fluxes in intact cells [1–3] To avoid such interferences, many authors have resorted to using non metabolizable synthetic amino acid analogs or to blocking amino acid catabolism by specific inhibitors [4–9]

The development of techniques for the isolation of plasma-membrane vesicles with transport activity [10–12] has added another tool for the study of amino acid transport at the hepatic level One attractive feature of the model is that, being free of cellular metabolic activity, it allows the unrestricted testing of any of the natural amino acids Liver plasma-membrane vesicles have been shown to retain the stimulation of amino acid transport induced in intact cells by cyclic AMP [13], glucagon [14], diabetes [15] and starvation [16]

In the first part of the work reported here, we present evidence for the maintenance of stimulated transport activity in plasma-membrane vesicles derived from the liver of rats adapted to high-protein feeding, a nutritional situation that enhances liver amino acid transport capacity [17,18]

The second part of the work was designed to investigate whether, as in intact hepatocytes [17], the stimulation of amino acid transport induced by high-protein feeding could be accounted for by a specific

Correspondence G Bourdel, Centre de Recherche sur la Nutrition, C N R.S, 9, Rue Jules Hetzel, F-92190 Meudon, France

stimulation of system A mediation To distinguish between system A- and system ASC-mediated transport, the method most widely used is through the use of *N*-methylaminoisobutyric acid (MeAIB) Being a substrate specific for system A, this non metabolizable analog allows the evaluation of both systems, system ASC being defined as the Na^+-dependent uptake retained in presence of excess MeAIB [4–6] For reasons presented below such strategy proved not to be feasible under our experimental conditions We thus turned to other discriminatory means, namely to the use of lithium as the driving ion and to the lowering of the pH of the incubation medium Although there is still some debate on the subject, system A is generally viewed as having a strict Na^+-dependency and as being inhibited at pH values below 7 0 [1,9,19–21]

In the last part, Na^+-dependent active transport of alanine and of serine was assayed in the presence of other competitor amino acid added in graded concentrations, roughly covering the range of physiological variations observed at the portal level Such a situation represents an approach closer to what actually occurs in vivo where portal total amino acidemia exhibits important quantitative variations related to digestive activity and levels of protein intake [22–24]

Materials and Methods

1 Materials

L-[G-^{3}H]Alanine, L-[G-^{3}H]serine, *N*-methylamino[1-^{14}C]isobutyric acid and D-[1-^{14}C]mannitol were purchased from New England Nuclear Nitrocellulose filters (pore size 0 45 μm) were from Sartorius (Palaiseau, France) and L-amino acids from Merck (Nogent-sur-Marne, France)

2 Animals and diets

Adult male rats of the Wistar strain weighing 200 g at the start of the experiments were used They were kept in individual cages under controlled conditions of light (12/12 light-dark cycle) and temperature (24° C) while fed either a low- or a high-protein diet The low-protein diet had the following composition (% w/w) casein (13), sucrose (20), maize-starch (52), peanut oil (8), cellulose (2), vitamin mixture (1), salt mixture (4) The high-protein diet contained casein (80), maize-starch (5), no sucrose, the other components being present in amounts identical to those in the low-protein diet After 4 weeks on either one of the 2 diets, the animals were killed by decapitation without anesthesia in state of prandial rest, 3 (L + 3), 6 (L + 6), and 9 (L + 9) hours after the onset of the light-period In each experiment 8 to 10 rats fed either one of the two diets were used. The reliability of the technique was ascertained in two ways. First L + 3 and L + 9 experiments were duplicated 5 months (L + 3) and 1 year (L + 9) apart Second, in an additional experiment the liver of each animal was cut in two halves processed in exact parallel except that for one half, homogenization and first two rounds of centrifugation were performed in presence of aprotinin (0 05 U/ml) and of phenylmethanesulfonyl fluoride (PMSF 1 mM)

To simplify the presentation of the results and the discussion, vesicles prepared from the liver of low-protein-fed rats will be referred as LP vesicles, those from the liver of high-protein-fed rats as HP vesicles

3 Preparation of liver membrane vesicles

Vesicles were prepared following a method which combines differential centrifugation and purification on a sel-forming Percoll gradient, all preparative steps being performed in iso-osmotic conditions at 4° C [25] The livers were quickly excised and minced in 5 vol of cold buffer (0 25 M sucrose, 0 2 mM $CaCl_2$, 10 mM Hepes-KOH, pH 7 4) to which 0 02% lithium azide was added Homogenization was performed in a Dounce homogenizer After filtration through a nylon cloth the homogenate was diluted 4-fold with buffer to which EDTA was added to achieve a final concentration of 1 mM It was centrifuged at 30 000 × *g* for 20 min The supernatant (S_1) when not used for enzyme assays was discarded The pellet was resuspended in buffer with EDTA and centrifuged at 700 × *g* for 10 min The supernatant (S_2G) was collected The pellet was resuspended in buffer with EDTA and centrifuged again as above The supernatant (S_2G) resulting from this second round of centrifugation was saved while the pellet (P_1) was discarded when not assayed for enzyme activity The two collected supernates (S_2G) were pooled and mixed with isotonic Percoll (Pharmacia, Bois d'Arcy, France) to give a final concentration of 11% The mixture was centrifuged at 30 000 × *g* for 30 min The membrane fraction was collected from the top of the gradient after exclusion of the first 1 5 ml, by pumping Maxidens (Nyegard, Oslo, Norway) into the bottom of the centrifuge tube for 5 min at a flow rate of 2 ml/min with a peristaltic pump (LKB, Orsay, France) The membrane fraction was washed with EDTA-free buffer (0 25 M sucrose, 0 2 mM $CaCl_2$, 10 mM Hepes-KOH, pH 7 4) with three cycles of suspension-centrifugation at 30 000 × *g* for 30 min The final pellet (M) was suspended in a small volume of the same EDTA-free buffer as above, divided in small portions, quickly frozen in liquid nitrogen and stored at −80° C Storage for up to 3 months did not cause any significant loss in transport or enzyme activity

4. Enzyme assays

5′-Nucleotidase (EC 3 1 3 5), Na,K-ATPase (EC 3 6 1 37), Mg-ATPase (EC 3 6 1 4), monoamine oxidase (EC 1 4 3 4), *N*-acetylglucosaminidase (EC 3 2 1 30) and glucose-6-phosphatase (EC 3 1 3 9) were assayed as de-

scribed previously [25] Phosphodiesterase 1 (EC 3 1 4 1) was assayed according to Touster et al [26], serine dehydratase (EC 4.2 1 13) and alanine aminotransferase (EC 2 6 1 12) were assayed according to Snell and Walker [27] and Segal and Matsuzawa [28] Protein in homogenates and plasma-membrane preparations was determined according to Peterson [29]

Protein yield, distribution and recovery of marker enzymes during the fractionation procedure were assessed in two preliminary experiments. In these experiments the processing buffer did not contain lithium azide In the first experiment (Expt I in Table I), 12 adult male rats fed a laboratory chow were used to follow the distribution of plasma-membrane markers (Na,K-ATPase, Mg-ATPase, phosphodiesterase 1, 5′-nucleotidase) In the second one, 10 rats fed either the 13% or the 80% casein diet (Expt II_A and II_B in Tables I and II) were used to assay plasma-membrane markers in parallel with the three enzymes associated with lysosomal (*N*-acetylglucosaminidase), mitochondrial (monoamine oxidase) and microsomal (glucose-6-phosphatase) membranes

5 Transport assays

A rapid mixing/filtration method was used For each assay 10 μl membrane vesicles was added to 40 μl buffer (0 25 M sucrose, 0 2 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM Hepes/KOH, pH 7 4) containing 1 μCi of either tritiated alanine or tritiated serine The reaction mixture contained initial gradients of either 100 mM NaSCN, 100 mM LiSCN or 100 mM KSCN and alanine or serine in concentration ranging from 0.1 to 5 mM The two amino acids were tested alone or in association with three different concentrations of a mixture of 15 amino acids (M1, M2, M3) obtained by adding appropriate amounts of the same concentrated solution to the incubation medium At the lowest concentration used (M1), the amino acid millimolar final concentration was the following, L-proline (0 73), L-threonine (0.55), L-glutamine (0 42), L-glycine (0 33), L-valine (0 33), L-lysine (0.31), L-leucine (0 27), L-tyrosine (0 21), L-isoleucine (0 19), L-asparagine (0 17), L-phenylalanine (0.13), L-arginine (0 09), L-methionine (0 09), L-aspartate (0 06) When alanine was the amino acid to be tested, the mixture contained L-serine 0.60 mM When serine was tested, 1 0 mM alanine was added in place of serine, so that total final concentration of amino acids in the incubation medium was 4 48 and 4.88 mM for M1, 8 95 and 9 75 mM for M2, 13.44 and 14 63 mM for M3 depending on the substrate in use In all cases, the components of the incubation medium were adjusted to constant initial concentrations of Na^+ and K^+ Constant osmolarity was achieved by varying the amount of sucrose in the incubation medium Reactions were carried at 25 °C and stopped after 5 s by addition of cold stop solution (0 25 M sucrose, 100 mM NaCl, 0 2 mM $CaCl_2$, 10 mM Hepes-KOH, pH 7 4) [10,25] In each experiment (L + 3, L + 6, L + 9), the assay were performed four times in triplicate on pooled samples of plasma-membrane vesicles prepared from either 13% or 80% casein-fed animals

Results

1 Distribution of protein and marker enzyme activities during plasma-membrane purification in preliminary experiments

In Expt I (Table I) no significant loss of protein or of enzyme activity was observed during the first steps of plasma-membrane preparation. 95% of protein initial

TABLE I

Protein and marker enzyme recoveries in preliminary experiments

Values are given in percentage of total homogenate protein or enzyme activity ± S E For H, values in brackets indicate mg protein/g liver, or μmol/min per g liver In Expt I rats were fed chow, in Expt II_A they were fed the 13% casein diet while II_B animals received the 80% casein diet Note the 53% increase in Na,K-ATPase in homogenates from rat II_B fed the high-protein diet when compared to II_A

Fractions	Protein			Na,K-ATPase			Mg-ATPase			5′-Nucleotidase		
	Expt I	Expt II_A	Expt II_B	Expt I	Expt II_A	Expt II_B	Expt I	Expt II_A	Expt II_B	Expt I	Expt II_A	Expt II_B
H	100 (192 ±6)	100 (183 ±6)	100 (213 ±6)	100 (2 3 ±0 3)	100 (2 8 ±0 4)	100 (4 3 ±0 2)	100 (20 ±0 4)	100 (27 ±0 3)	100 (34 ±1 7)	100 (11 ±0 5)	100 (11 ±0 5)	100 9 ±0 4)
S1	43±1			9±2			10±1			19±1		
P1	23±3			20±5			31±2			20±2		
S_2G	29±2	37±2	44±2	53±7	57±12	69±12	44±2	56±3	65±4	65±5	67±3	69±2
Total	95±2			85±14			85±4			103±5		
M	2 7 ±0 3	3 4 ±0 2	2 8 ±0 2	30±4	26±5	24±2	8±1	6±0 4	6±1	22±2	25±1	21±2
M/S_2G (%)	9±2	9±1	6±1	59±5	45±3	37±4	19±1	11±1	9±1	32±3	36±2	31±3

TABLE II

Recovery and relative specific activity of lysosomal, mitochondrial and microsomal marker enzymes in plasma-membrane preparations from low-protein fed (II_A) and high-protein-fed (II_B) rats

Fraction	*N*-Acetylglucosaminidase		Monoamine oxidase		Glucose-6-phosphatase	
	Expt II_A	Expt II_B	Expt II_A	Expt II_B	Expt II_A	Expt II_B
S_2G (% H)	96 ±10	96 ±11	102 ±13	103 ±17	41 ±3	45 ±6
M (% H)	12± 02	10± 01	180± 02	142± 02	38±04	41±04
RSA	02	04	04	05	12	14

amount, 85% of Na,K-ATPase and Mg-ATPase, 100% of 5′-nucleotidase homogenate activity was recovered by summing up S1, P1 and S2G contents The balance sheet could not be drawn further due to the unreliability of the assays performed in the Percoll gradient fraction remaining after recovery of fraction M In fraction M, the highest yield was that of Na,K-ATPase which amounted to 30% of the homogenate activity or 60% of that in S2G In Expt II_A and II_B, the amount of Na,K-ATPase recovered in S2G compared well with that obtained in Expt I However, greater losses occurred between S2G and M leading to lower final recovery (45 to 37% for MII_A and MII_B, respectively) Table II shows that contamination by lysosomal and mitochondrial membranes was minimal in these fractions Given the high levels of *N*-acetylglucosaminidase and of monoamine oxidase still present in fraction S2G, the Percoll gradient step appeared very efficient in the clearing off of these two types of contamination It was less so in the case of endoplasmic reticulum as fractions MII_A and MII_B still contained a sizeable proportion of glucose-6-phosphatase original activity

2 Protein content and enzyme activities in the liver and in plasma-membrane preparations from rats fed either the low- or the high-protein diet and killed at different times during light-time

Intake of the high-protein diet significantly increased liver protein content, serine dehydratase and alanine aminotransferase activities (Table III) The adaptative response was most striking with serine dehydratase which increased 40-fold over the level observed in rats fed the low-protein diet, while alanine aminotransferase activity was multiplied only by 4 The nutritional impact on the activity of the enzyme marker of the basolateral domain, Na,K-ATPase, was also clear-cut In the high-protein fed group, the enzyme activity was increased by 60% as compared to that in the low-protein fed one Unlike Na,K-ATPase, the other three enzymes which are predominantly located in the canalicular area, were not significantly affected by the dietary change

In the present experiments the value of Mg-ATPase activity in homogenate was drastically reduced (by about 70%) when compared to those obtained in Expt I and Expt II (Table I) This was due to the addition of 0 02%

TABLE III

Liver protein content (mg/g) and enzyme activities (μmol/min per g) in rats fed either a low (13% casein) or a high (80% casein) protein diet

First column L+3, L+6 and L+9 refer to rats killed 3, 6 or 9 hours after the onset of the light period, between brackets number of rats Values are means of individual assays ± S E

	Protein (mg/g)	Serine dehydratase	Alanine amino-transferase	Na,K-ATPase	Mg-ATPase	5′-Nucleo-tidase	Phospho-diesterase I
13% Casein diet							
L+3 (16)	154±4	116$^+$	135$^+$	$320\pm015_a$	738±037	$103\ \pm04_a$	$208\pm012_a$
L+6 (8)	164±6	188±15	163±15	$255\pm017_b$	863±033	$108\ \pm04_a$	$248\pm012_B$
L+9 (18)	163±7	161±04	140±13	$227\pm013_c$	843±050	$120\ \pm04_b$	$400\pm025_c$
80% Casein diet							
L+3 (16)	204±4**	480$^+$	457$^+$	$447\pm022_a$**	850±035	$997\pm018_a$	$177\pm010_a$*
L+6 (8)	200±7**	537±31**	544±29**	$443\pm023_a$**	810±027	$108\ \pm04_a$	$300\pm020_b$*
L+9 (18)	196±7**	406±30**	640±32**	$363\pm020_b$**	815±040	$116\ \pm04_b$	$482\pm040_c$

$^+$ Assayed on pooled samples

* and ** Significant differences (* $P < 005$, ** $P < 001$) with 13% casein-feeding

a, b, c Different subscripts indicate significant differences ($P < 001$) in relation to killing-time

TABLE IV

Protein content (mg/ml), protein yield, enzyme activity (μmol/min/ml), recovery (% homogenate activity) and purification (relative specific activity) in plasma-membrane preparations

Values are the means ± S E of the five experiments performed at L+3, L+6 and L+9

	Protein	Na,K-ATPase	Mg-ATPase	5′-Nucleotidase	Phosphodiesterase 1
13% Casein diet					
mg or U/ml	6 4±0 5	0 83±0 02	1 37±0 17	2 75±0 20	0 33±0 03
R (%)	3 2±0 2	25 9 ±2 2	14 4 ±0 7	20 4 ±1 0	10 1 ±0 10
RSA		7 6 ±0 9	4 8 ±0 7	6 6 ±0 6	3 3 ±0 3
80% Casein diet					
mg or U/ml	5 1±0 5	1 58±0 03	1 17±0 08	2 57±0 33	0 41±0 02
R (%)	2 1±0 1	28 6 ±0 7	11 7 ±1 0	18 8 ±2 2	12 3 ±3 2
RSA		14 0 ±1 1	6 0 ±0 9	9 5 ±0 5	6 2 ±1 7

lithium azide to the processing buffer This addition had only a slight effect on Mg-ATPase activity measured in fraction M and no effect on Na,K-ATPase activity either in homogenate or fraction M Table IV presents protein yields, marker enzyme concentrations, levels of recovery and purification in plasma membrane preparations Since no significant time-related changes in enzyme activity were detected in plasma-membrane preparations, data from L + 3, L + 6, and L + 9 experiments were pooled for presentation in Table IV In spite of higher protein content in homogenate (see Table III), protein yield was significantly lower in plasma-membrane preparations derived from the liver of rats fed the high-protein diet This resulted in a better purification of all marker-enzymes Of importance was the constancy of the pattern of recovery irrespective of the nutritional condition, recovery of Na,K-ATPase was higher (25 to 30%) than that of the canalicular enzymes Mg-ATPase and phosphodiesterase 1 (14 to 10%) with that of 5′-nucleotidase being intermediate (19%) As in homogenates, the activity of Na,K-ATPase was significantly higher in HP vesicles (1 58 versus 0 83 μmol ATP hydrolyzed/min per ml) It thus appears that Na,K-ATPase activity in purified preparations was representative of the initial activity in the homogenate However, in the 80% casein-fed group, Na,K-ATPase activity in homogenates was increased 1 5 ± 0 7-fold over that observed in the 13% casein-fed animals In fractions M, the ratio was increased to 1 84 ± 0 9-fold, suggesting greater enrichment in basolateral membrane (+20% compared to LP) in HP preparations As a correction for this technical bias, it was decided to express the amino acid transport activity in terms of Na,K-ATPase activity Other reasons based on physiological considerations that will be developed later, concurred to the justification of this mode of expression

3 Na^+-dependent active transport of alanine and of serine present as sole substrate in the incubation medium

Uptake of alanine or serine was measured over the same range of concentration (0 1 to 5 mM) In presence of 100 mM KSCN uptake increased linearly with substrate concentration (not shown) Na^+-dependent active transport calculated as the difference between uptakes in presence of 100 mM NaSCN and 100 mM KSCN on the contrary, was clearly saturable as shown in Fig 1 When transport activity, as in this case, is expressed per unit Na,K-ATPase, the striking parallelism of alanine and of serine Na^+-dependent active transport, is clearly evidenced It is also clear that the two amino acids are taken up with equal affinity and velocity by both types of plasma-membrane preparations Eadie-Hofstee plots of the data in Fig. 1, yielded superimposable single straight lines for each amino acid and each nutritional situation ($P < 0\,01$ in all cases) This means that in vesicles derived from rats fed the 80% casein diet, transport capacity rose in parallel with Na,K-ATPase activity That high-protein feeding induced an intrinsic

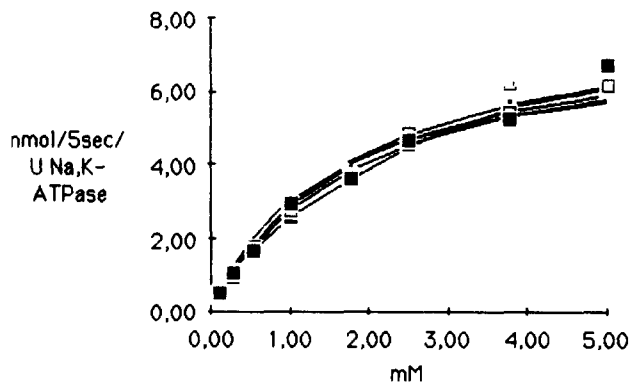

Fig 1 Active Na^+-dependent transport of alanine and of serine by vesicles derived from the liver of rats fed either low- or high-protein diets Transport activity, calculated as the difference between uptake in the presence of Na^+ gradient and uptake in the presence of K^+ gradient is expressed as nmoles transported for 5 s per unit Na,K-ATPase in vesicle preparations (0 83 and 1 58 U/ml for low- and high-protein feeding, respectively) Values are the means of 60 to 75 determinations S E were less than 5% of the corresponding mean value □, Alanine, ■, serine transported by vesicles from rats fed the 13% casein diet, △, alanine, ▲, serine transported by vesicles from rats fed the 80% casein diet The hyperbolas were drawn from $V = V_{max}\, S/(K_m + S)$, V_{max} and K_m were calculated from Eadie-Hofstee plots

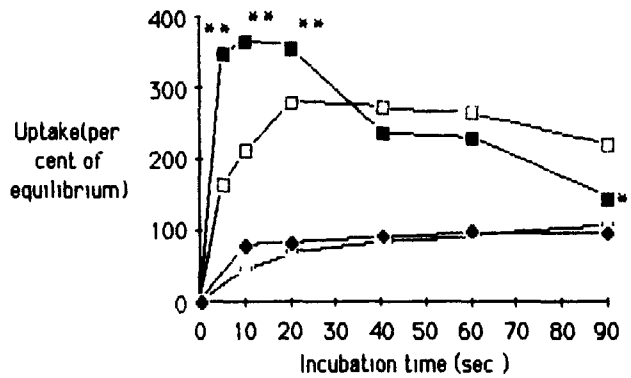

Fig 2 Time-course of alanine uptake in sodium- or potassium-containing medium Plasma-membrane vesicles from rats fed the 13% casein (open symbols) or the 80% casein (closed symbols) diet were incubated for the time indicated in medium containing either 100 mM NaSCN (□, ■) or 100 mM KSCN (◇, ◆) and 0 25 mM alanine Each point represents the mean of the six determinations from a representative experiment (L+6) Significant difference between low- and high-protein feeding is indicated by * ($P < 0\,05$) and ** ($P < 0\,01$) Uptake at equilibrium (60 min) was similar in both groups $8\,56 \pm 0\,6$ and $9\,06 \pm 1\,4$ pmol/10 μl for low- and high-protein feeding, respectively

stimulation of amino acid transport is best illustrated by comparing the time-course of Na^+-dependent uptake observed in the two types of vesicles preparations (Fig 2) In the figure, alanine intravesicular concentration at each time-point is expressed in relation to the volume of the intravesicular space defined by the respective levels of alanine accumulation at equilibrium (60 min) HP vesicles had a faster and a greater concentrative power than LP ones Maximal alanine concentration (3.8-fold) was achieved in less than 5 s

As mentioned before, duplicate experiments were performed at L + 3 and L + 9 A close agreement was obtained for alanine transport both at L + 3 and L + 9 Duplication for serine transport was more at variance, particularly at L + 3 for LP vesicles (Fig 3) and L + 9 for HP ones (not shown)

4 MeAIB inhibitory capacity and transport in plasma membrane vesicles

In experiments with hepatocytes, whether freshly isolated or cultured, most authors use 100-fold excess MeAIB to evaluate ASC transport In the new context provided by the experimental model used here, the relative amount of MeAIB most appropriate for this purpose had to be determined Therefore the inhibitory effect of 5- to 500-fold excess MeAIB on 0 1 to 5 0 mM alanine transport in Na and Li medium was first assayed (Table V). MeAIB concentration had to be at least 20-fold that of alanine to exert a sizeable inhibition of Na^+-driven transport However, from there on, there was no clear stabilization of the inhibitory effect Moreover, even with as low as a 20-fold excess some kind of unspecific inhibition could already be suspected as lithium-driven alanine transport was also inhibited

In the face of these unexpected difficulties, we decided to assess MeAIB transport directly The time-

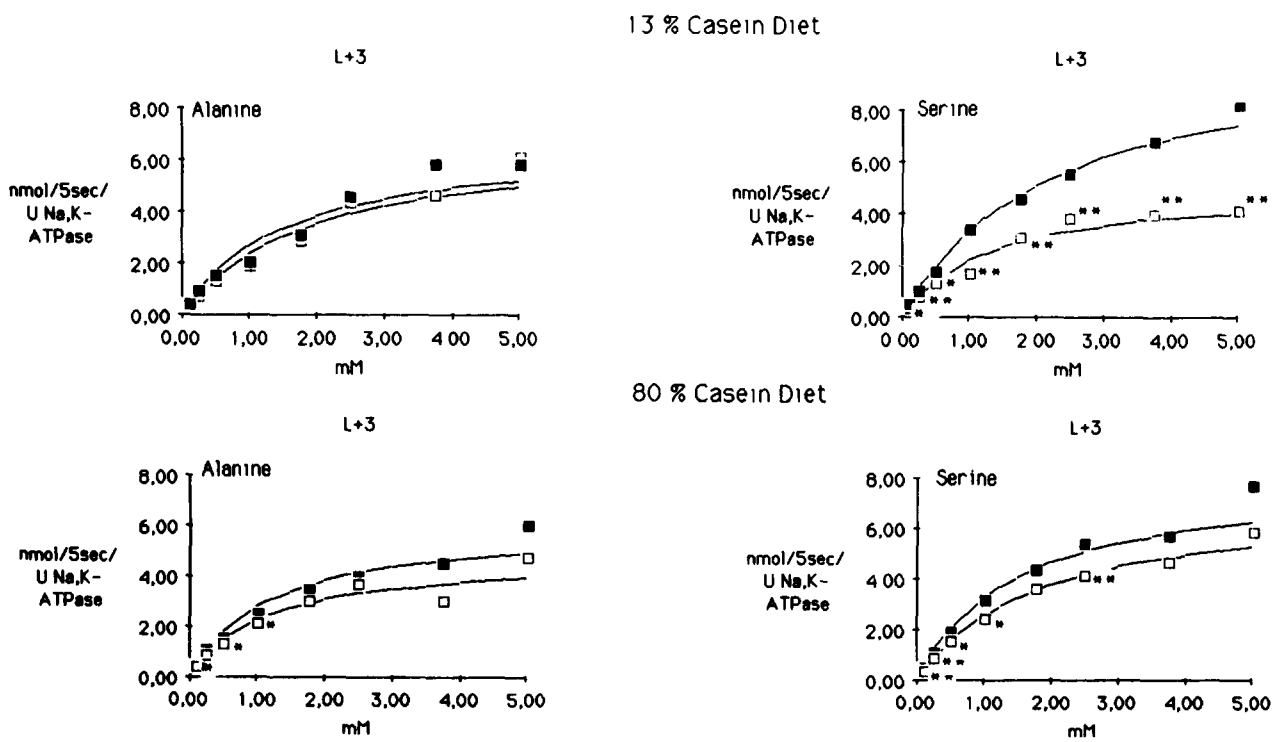

Fig 3 Active Na^+-dependent transport in duplicate experiments performed at L+3 □, Expt 1, ■, Expt 2 * ($P < 0\,05$), ** ($P < 0\,01$) indicate significant difference between experiments The transport activity is expressed as in Fig 1

TABLE V

Percent inhibition of alanine active transport by excess MeAIB

Numbers in brackets indicate the number of assays MeAIB addition (2 to 50 mM) was made at the expense of the sucrose content of the incubation buffer (basal composition 0 25 M sucrose, 0 2 mM $CaCl_2$, 10 mM $MgCl_2$, 10 mM Hepes-KOH, pH 7 4) The inhibitory effect of MeAIB on alanine transport was tested over the usual range of alanine concentration (0 1 to 5 0 mM) in presence of initial gradients of 100 mM, Na, Li or KSCN, and is expressed in percentage of alanine transport tested alone at corresponding concentrations For a given MeAIB/alanine ratio, several combinations of concentrations of inhibitor/substrate were tested Uptake was measured over 5 s at 25 °C The reaction was started by addition of 10 µl vesicles to 40 µl incubation medium (both prewarmed) and was terminated by addition of 1 ml cold stop solution (0 25 M sucrose, 100 mM NaCl, 0 2 mM $CaCl_2$, 10 mM Hepes-KOH, pH 7 4) The whole content of the tube was quickly filtered through a nitrocellulose filter (pore size 0 45 µm), washed with 3 ml cold stop solution, dried and counted

		Percent inhibition at [MeAIB]/[alanine]						
		5	10	20	50	100	200	500
	Na-mediation	(36)	(36)	(30)	(24)	(18)	(12)	(6)
	Li-mediation	(12)	(12)	(12)	(12)	(9)	(6)	(3)
13% Casein diet	Na medium	15±8	29±10	43±15	54±10	71± 8	75± 5	81
	Li medium	0	0	10±10	15±10	14±17	61±20	53
80% Casein diet	Na medium	10±8	31± 7	41±11	62± 6	75± 7	85± 9	85
	Li medium	0	0	4± 3	16± 7	25± 3	38± 1	51

course of the intravesicular accumulation of either MeAIB, alanine, or serine added at the same concentration (0 25 mM) to incubation medium containing either 100 mM NaSCN or 100 mM KSCN, was assayed in parallel and compared with that of 0 25 mM mannitol which is considered to diffuse freely through the plasma-membrane The results are presented in Fig 4 In LP vesicles no concentrative uptake of MeAIB in Na medium was observed In HP vesicles an overshoot was seen but it was half of those observed with alanine and serine. It was also much slower maximal accumulation was at 20 s when efflux already took precedence over influx in the case of the two natural amino acids The low velocity of MeAIB crossing of the plasma membrane also documented in experiments with hepatocytes [14,19–21,30], probably explains the low efficiency with which MeAIB was driven into the vesicles Dissipation with time of the Na^+-gradient is the main limiting step in the experimental system used here [10,31]. The use of SCN^-, selected in preference to less lipophilic anions [14,32] because of the low transport capacity of the LP vesicles, may have enhanced the disparity between MeAIB transport and that of alanine and of serine

5 Lithium-driven alanine and serine active transport

The effect of substituting Li^+ for Na^+ on active transport of alanine and serine, is presented in Fig 5 In LP vesicles, Li^+ induced a drastic reduction in transport capacity ($P < 0\ 01$) at all substrate concentrations tested Li^+-activated transport was roughly half that supported by Na The situation was totally different with HP vesicles. Li^+ was clearly less effective than Na^+ in promoting active transport at low alanine and serine concentrations (0 1 to 0.25 mM) At these concentrations transport capacity was reduced by half as

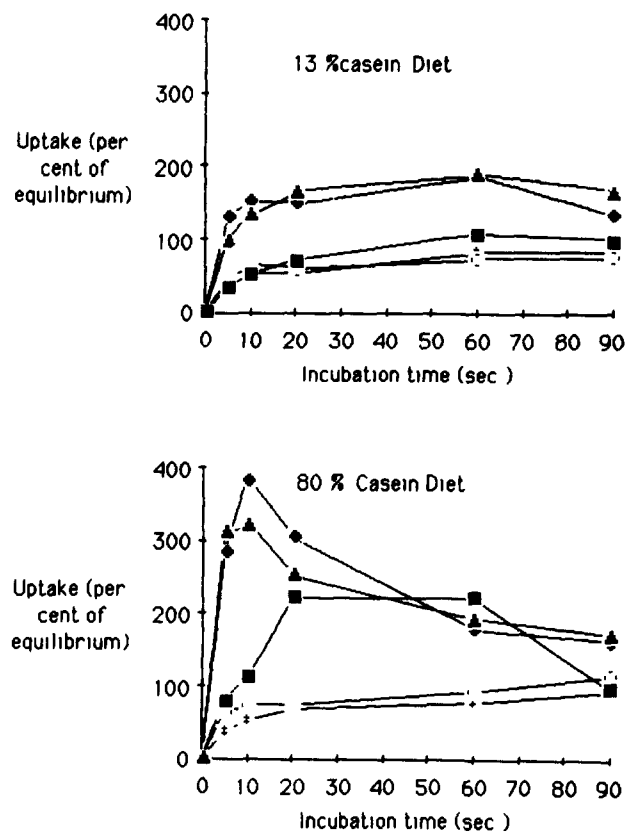

Fig 4 Time-course of uptake of different substrates by LP vesicles (13% casein diet) and HP vesicles (80% casein diet) At time zero 10 µl vesicles were added to 40 µl incubation medium containing either 100 mM NaSCN (closed symbols) or 100 mM KSCN (open symbols) and 0 25 mM alanine (◆, ◇), serine (▲), MeAIB (■), mannitol (*) Incubations (25 °C) were terminated at the time indicated on the abscissa by addition of 1 ml cold stop solution Uptake at equilibrium (pmol/10 µl) was as follows LP vesicles alanine 14 7±0 7, serine 16 9±0 4, MeAIB 15 3±0 7, mannitol 10 3±1 0 HP vesicles alanine 12 7±0 3, serine 17 1±0 5, MeAIB 16 4±0 9, mannitol 10 4±0 9

with LP vesicles The gap between Na^+- and Li^+-driven transport tended to close with the rising of substrate concentrations so that the differences became unsignificant from 2 5 mM up In HP vesicles, all liver alanine and/or serine carriers were able to accept Li^+ even though the affinity towards the substrates was lowered At this point, it was thought necessary to ascertain that this effect was not a procedural artifact due to poor control of proteolysis during sample preparation it has been shown that, at least for certain types of Na^+-channels, mild proteolysis can affect ion-selectivity [33] Therefore, the ion-selectivity of vesicles prepared with or without proteinase inhibitors and derived from the same livers were compared In inhibitor-treated LP vesicles, Li^+ still failed to support as much transport as Na^+, while with HP vesicles transport kinetics with Li^+ or Na^+ were undistinguishable except at 0 1 mM substrate concentration ($P < 0\ 01$) as seen in Fig 6 which illustrates results for alanine The same observation was made with serine as well (not shown)

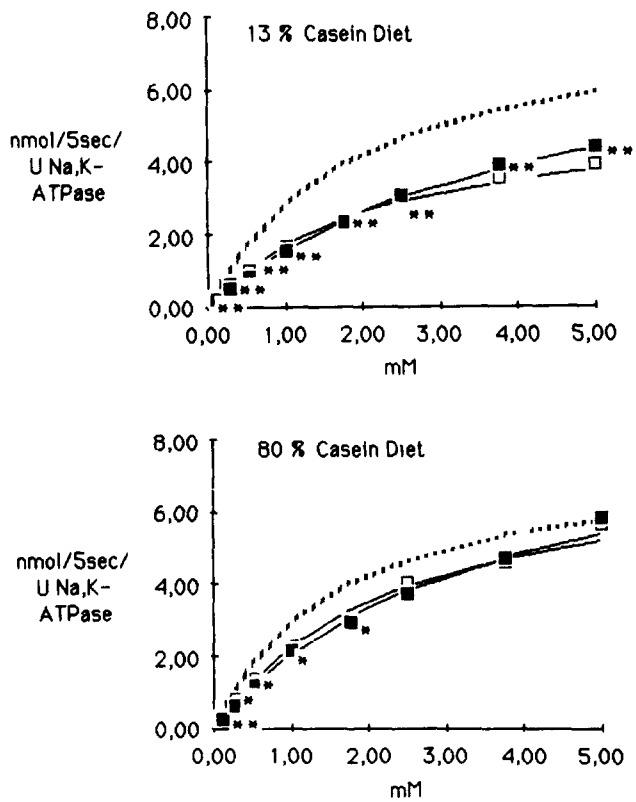

Fig 5 Li^+-dependent active transport of alanine and of serine by LP vesicles (13% casein diet) and HP vesicles (80% casein diet) Active transport corresponds to 5 s uptake in 100 mM LiSCN minus uptake in 100 mM KSCN medium The values are the means of 60 to 75 determinations of alanine and serine transport from five experiments □, Alanine, ■, serine The hyperbolas were drawn from $V = V_{max}\ S/(K_m + S)$ V_{max} and K_m were calculated from Eadie-Hofstee plots The dotted line corresponds to Na^+-driven active transport of alanine and serine computed together given as reference * ($P < 0\ 05$), ** ($P < 0\ 01$) indicate significant differences (calculated by analysis of variance) between experimental values for Na^+- and Li^+-dependent transport

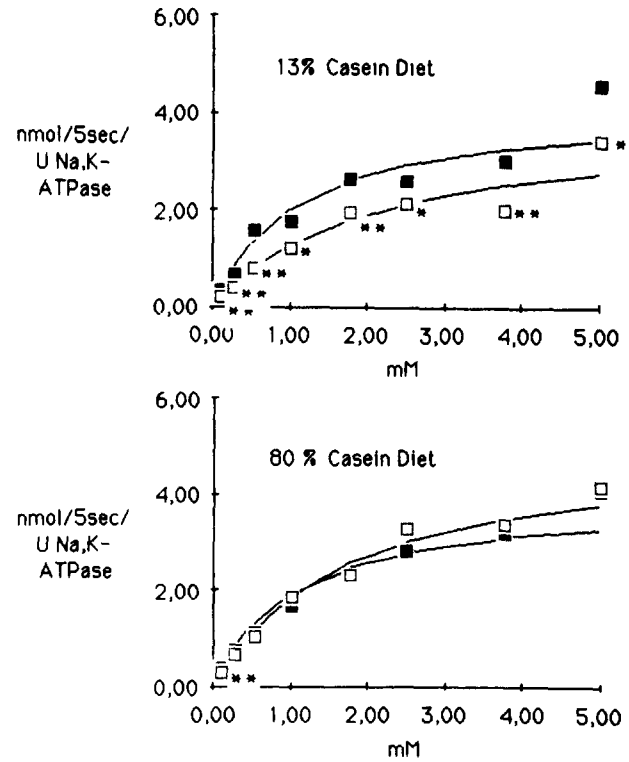

Fig 6 Na^+- and Li^+-dependent active transport of alanine by LP vesicles (13% casein diet) and HP vesicles (80% casein diet) prepared in the presence of proteinase inhibitors ■, Na^+-driven transport, □, Li^+-driven transport The values are the means of 12 determinations * ($P < 0\ 05$), ** ($P < 0\ 01$) indicate significant differences between Na^+- and Li^+-driven transport at corresponding substrate concentration Na,K-ATPase concentration 0 62 and 1 13 U/ml in LP and HP vesicles, respectively

6 Effect of pH on active transport capacity

With LP vesicles, lowering of the external pH to 6 5, reduced by half Na^+-driven transport at all substrate concentrations (Fig 7) For Li^+-driven transport already curtailed by half at pH 7 5, no further reduction was observed In HP vesicles, Na^+- as well as Li^+-driven transport was reduced at low pH Compared to Na^+-driven transport at pH 7 5, the decrease (40%) was of the same order as the one observed with LP vesicles Thus active transport in HP vesicles, whether Na^+- or Li^+-driven, was as sensitive to the lowering of the pH as it was in LP vesicles The only difference between the two types of vesicles was in the degree of Li^+ acceptance at pH 7 5

7 Alanine and serine Na^+-dependent active transport in presence of other amino acids

Active transport of alanine and serine in concentrations ranging from 0 25 and 5.0 mM was tested in presence of three graded concentrations of the same amino acid mixture Measures were performed in situa-

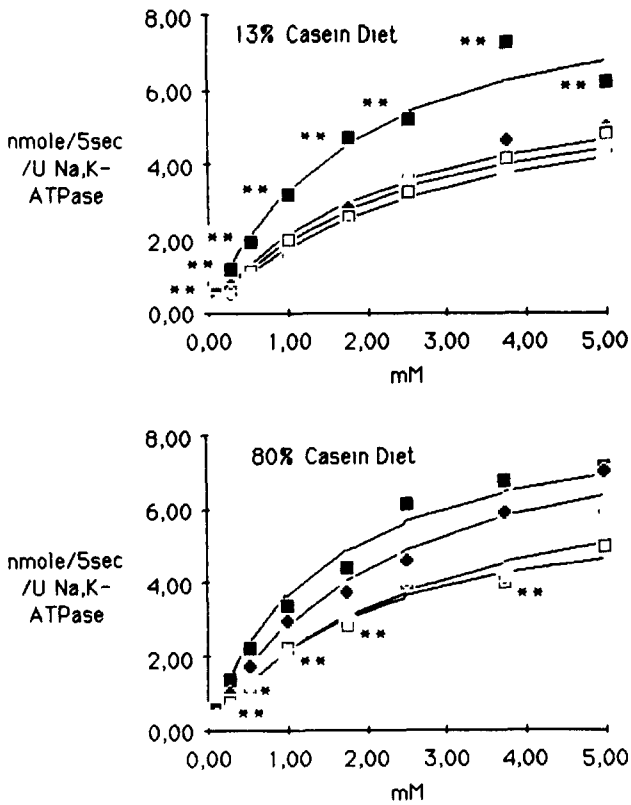

Fig 7 The pH-dependent active transport of alanine by LP vesicles (13% casein diet) and HP vesicles (80% casein diet) Lowering of external pH from 7 5 to 6 5 was obtained by monitoring the pH of the buffer component (10 mM Hepes-KOH) of the incubation medium (0 25 M sucrose, 0 2 mM $CaCl_2$, 10 mM $MgCl_2$) The values are the means of 24 determinations from two experiments (rats killed at L+6 and L+9) Closed symbols correspond to incubation at pH 7 5, open symbols to incubation at pH 6 5 (□ ■, Na^+-, ◇ ◆, Li^+-driven transport) The significance of differences (* $P < 0\,05$, ** $P < 0\,01$) was tested by analysis of variance For LP vesicles the comparison was between Na^+-driven transport and the other experimental conditions taken together For HP vesicles the test was between Li^+-driven transport at pH 7 5 and transport at pH 6 5, whether driven by Na^+ or Li^+ Na,K-ATPase concentration 0 67 and 1 24 U/ml in LP and HP vesicles, respectively

tion of zero intravesicular concentration for both substrate and competitor amino acids

Substantial inhibition of alanine and serine active transport was observed both with LP and HP vesicles Owing to the composite nature of the competitor mixture, no kinetic analysis was attempted However the experimental design resulted in the testing of transport over a large range of relative concentration of substrate from less than 2% (0 25 mM alanine or serine + M_3) to more than 50% (5 0 mM + M_1) This allowed to see that the inhibitory pressure of the amino acid mixture was clearly dependent on the amino acid mixture concentration used in relation to the concentration of substrate and was the same in LP and HP vesicles as shown in Fig 8

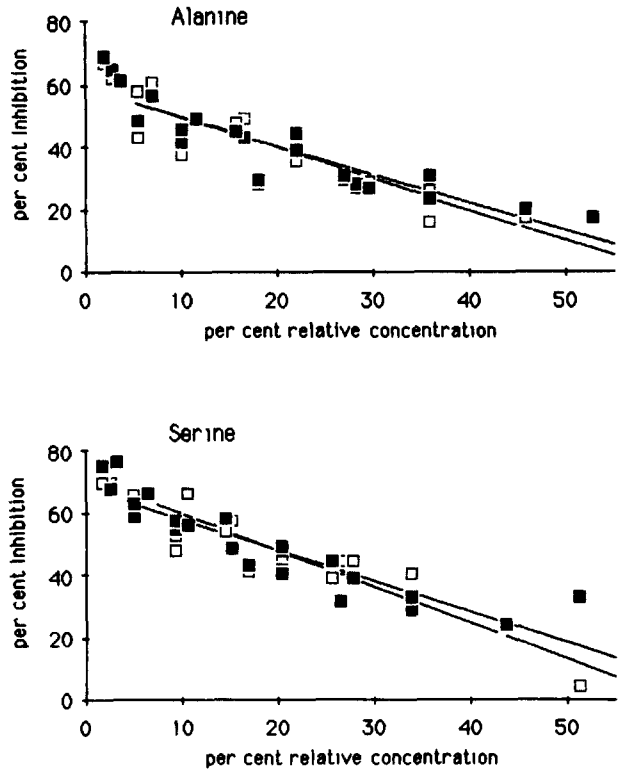

Fig 8 Percent inhibition of alanine and serine active transport as a function of relative concentration of substrate Values are the means of five experiments □, low-protein diet, ■, high-protein diet

Discussion

The method used to prepare plasma-membrane vesicles is rapid and does not require special equipment It allows the recovery of preparations enriched in vesicles derived from the basolateral domain of the hepatocyte plasma membrane and able to give reproducible estimates of amino acid transport capacity Two other procedures for the isolation of liver plasma-membrane vesicles, also based on Percoll gradient purification, have been published which give lower protein contamination but also lower yields of 5′-nucleotidase or Na,K-ATPase [11,12] However, yield is often a limiting factor in amino acid transport studies

Relationship between Na,K-ATPase and amino acid transport activity

Liver Na,K-ATPase activity was clearly increased by high-protein feeding Total liver activity rose from 30.2 μmol ATP hydrolized per min in low-protein-fed animals to 49 5 in high-protein-fed rats Likewise, the activity of the enzyme was increased 2-fold in vesicles derived from the liver of rats fed the high-protein diet This increase bore a direct relationship with the increase seen in homogenate since no more than 20% of it could be attributed to better enzyme recovery If Na,K-ATPase activity in vesicles closely reflects the activity in liver, it

can be assumed that the same occurs regarding amino acid transport activity In this case, it is clear that amino acid transport capacity rose following high protein feeding However, the increase paralleled that of Na,K-ATPase so closely that the relationship between the two systems was kept constant irrespective of the nutritional conditions This is important from a physiological point of view The Na,K-ATPase or Na,K pump, is unique in its ability to pump intracellular Na^+ ions in excess out of the cell against the physiological gradient that it so contributes to maintain Thus such an increase, in exact coordination with that of amino acid transport activity, should insure the full efficiency of stimulated liver transport in the rats receiving a high-protein diet Van Dyke and Scharschmidt [34] have presented evidence that cation pumping by Na,K-ATPase increases in response to the stimulation of alanine transport in hepatocytes In HeLa cells the relationship was found so tight as to suggest that amino acid transport is the major determinant of pump activity [35] Conversely, in normal fibroblasts cultured in presence of ouabain, amino acid transport is increased in parallel with Na,K-ATPase [36] However, the parallelism between changes in Na^+-pumping and amino acid transport is not obligatory For example, it was not observed by Pastor-Anglada et al [25] in 12-day pregnant rats In this case, alanine transport was increased without change in Na,K-ATPase activity The mechanisms of hormonal and of nutritional stimulation may thus be different Liver Na,K-ATPase is much higher in females (7 0 μmol ATP hydrolyzed per min per g liver in non-pregnant females fed a 17% protein diet) than in males (4 2 μmol in the males fed the high-protein diet) and might not be as readily inducible

Functional characteristics of alanine and serine active transport in LP and HP vesicles

High-protein feeding induced a stimulation of active transport which was of similar magnitude for alanine and for serine The increase occurred with no change in affinity toward the substrates as suggested by the finding of identical slopes in Eadie-Hofstee plots for LP and HP vesicles These results are consistent with an increase in the number of transport sites inserted at the plasma-membrane level

Probing active transport with Na^+ as the motive ion gives an overall evaluation in which the respective contribution of system A and ASC cannot be distinguished since both systems are dependent on Na-mediation It has been suggested that system ASC could be distinguished from system A by its tolerance for Li^+ as a substitute for Na^+ [9] and by its weak pH-dependency at pH above or equal to 6 5 [19–21,30] While the value of the first of these two criteria has been criticized [19], the second has gained wider acceptance

In LP vesicles, Li^+-driven active transport of alanine and serine whether measured at pH 7 5 or at pH 6 5 was of comparable magnitude and represented 50% of Na^+-driven transport at pH 7 5 If transport at pH 6 5 is equated to transport through system ASC, then the suggestion by Edmonson et al [9] that Li^+ may be used to elicit directly the contribution of liver ASC system seems warranted Accordingly and in agreement with what has been observed with hepatocytes [4], alanine transport in LP vesicles (and that of serine as well) distributed equally between system A (strict Na^+ dependency, pH sensitivity) and ASC (Li^+ tolerance, pH insensitivity)

This is no longer true when results from HP vesicles are considered In this case, the pH-sensitive component (A-like) appeared to have a high degree of Li^+ acceptance Kilberg et al [4] have mentioned that stimulation of Na^+-sustained AIB transport which in cultured hepatocytes is a specific test-substrate for system A, often went with an increase in the acceptance of a Li^+ mediation Our results so far agree with this conclusion in that the broadening of ion-acceptance in HP vesicles was observed in conjunction with a stimulation of overall Na^+-driven transport For the pH-insensitive, Li^+-tolerant component assignment to ASC mediation seems justified Its contribution (60%) to total Na^+-dependent transport remained similar to the one observed with LP vesicles It increased in parallel with overall Na^+-driven transport Our results thus suggest that, in the long-term, high-protein feeding can stimulate amino acid transport through system ASC as well as through system A System ASC is generally considered to be unresponsive to nutritional as well as to hormonal stimuli [1,2,5–6] Results that challenge this paradigm can be found Kristensen et al [8] observed an increase in alanine transport resistant to MeAIB inhibition in hepatocytes from starved rats Pastor-Anglada et al [25] also found an increase in MeAIB-resistent alanine transport in liver plasma-membrane vesicles from female rats at mid-pregnancy Moule and Bradford [37] recorded a stimulation of ASC-mediated transport for alanine in hepatoctyes stimulated by cAMP Increase in ASC capacity was also observed in hepatocytes freshly isolated from diabetic [38] and adrenalectomized [39] rats

Our studies with liver plasma-membrane vesicles confirm that, in the long-term, high protein feeding increases the number of carriers for alanine and serine transport The properties exhibited by these carriers preclude their unquestionable classification as A or ASC

Alanine and serine active transport in presence of other amino acids

In presence of other amino acids, Na^+-dependent transport of alanine and of serine was inhibited in plasma-membrane preparations The degree of inhibition, similar for both amino acids and in both nutri-

tional situations, was a function of the relative concentration of the substrate This is of interest when viewed in perspective with the type of amino acid variations observed at the portal level in relation to the composition of the diet and the stage of digestive activity Indeed, in vivo, while portal aminoacidemia is highly variable in absolute terms, its amino acid profile in terms of relative concentration of individual amino acids, remains rather stable For instance in rats fed a 13%casein diet, amino acid total concentration in the portal vein can rise from 3 (light-period) to 5 mM (night-period) Ingestion of a protein-rich meal can bring it from 4 to 8 mM in less than 3 hours [22] Portal amino acid concentrations as high as 14 and 20 mM have been reported in rats receiving 50% or 90% casein in their diet [23,24] In all these situations, notwithstanding the huge differences in portal amino acid concentration, alanine contribution represented 15 to 20% and serine 6 to 12% of total amino acids Our results suggest that whatever the nutritional status, alanine transport will be less impeded than that of serine because alanine is the amino acid found in highest concentration in the portal vein However, the conclusion that hepatic transport is stimulated by high-protein feeding still holds because vesicles from high-protein-fed animals, being enriched in transport sites, are able to take up greater amounts of alanine and of serine

The question as to whether transport across the membrane is rate-limiting for hepatic utilization of amino acids and particularly of gluconeogenic ones has often been discussed [8,40–43] Following high-protein intake, liver intracellular gluconeogenic amino acid concentration falls in vivo [22–24] In vitro alanine does not accumulate in hepatocytes from high-protein-fed rats when external alanine is kept around 2 mM, a level corresponding to the portal concentration observed after high-protein feeding [23,24,43] In the present work, adaptation to a high-protein diet resulted in a 4-fold increase in hepatic alanine aminotransferase activity while transport capacity was only doubled The imbalance was even greater with serine as serine dehydratase activity was multiplied by 40 Thus, our results can be regarded as adding support to the view that despite its capacity for adaptation, liver amino acid transport is a rate-limiting step for amino acid catabolism

Acknowledgments

The authors wish tot hank Dr A Girard-Globa and Dr M Pastor-Anglada for their valuable suggestions The expert secretarial assistance of M Fernandez is gratefully acknowledged These studies were supported by Institut National de la Santé et de la Recherche Médicale, grant CRE No 857001

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