Stimulation of amino acid transport into liver cells from rats adapted to a high-protein diet

Pierre FAFOURNOUX, Christian RÉMÉSY and Christian DEMIGNÉ
Laboratoire des Maladies Métaboliques, I.N.R.A.—Theix, 63110 Beaumont, France

(Received 24 November 1981/Accepted 9 March 1982)

After adaptation of rats to a 90%-casein diet, hepatic uptake of alanine is strikingly increased *in vivo*, with concomitant appearance of a concentration gradient favourable for uptake. With a high-protein diet, uptake of 2-aminoisobutyrate by isolated hepatocytes in the presence of various concentrations of substrates suggested induction of the A system (high-affinity system), whose emergence has been reported during starvation or after glucagon treatment. The other systems (ASC, L) were characterized: induction processes only affected the A system. Dibutyryl cyclic AMP addition resulted in an increase in 2-aminoisobutyrate transport at low substrate concentration, the response being greater after adaptation to a high-protein diet. Evidence is presented suggesting that the increased uptake of amino acids by the liver of rats fed on high-protein diets is obtained by developing favourable gradients and enhancing transport capacities. These adaptations allow sufficient amounts of amino acids to enter the liver, where accelerated metabolism plays a decisive role.

After intestinal absorption, amino acids have to pass through the liver before entering the general circulation. Liver extracts most of the absorbed amino acids when their availability is high, as in rats fed on high-protein diets (Rémésy *et al.*, 1978). Studies on amino acid transport into the liver with non-metabolizable analogues indicate that the transport systems of the liver cell membrane are closely similar to the A, ASC and L systems (Le Cam & Freychet, 1977) that have been previously characterized in Ehrlich cells (Christensen *et al.*, 1973). Both amino acid metabolism and transport could be influenced by dietary or hormonal factors (Le Cam & Freychet, 1976; Guidotti *et al.*, 1978; Canivet *et al.*, 1980).

During starvation (Fehlmann *et al.*, 1979), there is also an increase in amino acid transport resulting from the emergence of the A system, which increases the ability of hepatocytes to concentrate amino acids at low blood concentrations. According to Sips *et al.* (1980) and McGivan *et al.* (1981), at low alanine concentrations the transport of this amino acid appears to be rate-limiting for metabolism. When the availability of amino acids is raised after giving high-protein diets, another situation occurs, as there is a more favourable gradient for amino acid uptake (Rémésy *et al.*, 1978). At the very high alanine concentrations encountered in portal blood of these fed rats, the question arises whether alanine trans-

port is still enhanced by the induction of new transport systems. The aim of the present investigation was thus to characterize the responsiveness of the A, ASC and L systems after adaptation to a high-protein diet. In contrast with starvation, the induction of the A system by a high-protein diet was observed in the presence of a high availability of amino acids, but accelerated alanine metabolism must be more important than increased transport.

Materials and methods

Chemicals

2-Amino[1-¹⁴C]isobutyric acid (60 mCi/mol) and [¹⁴C]cycloleucine (60 mCi/mol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.); ³H₂O (1 mCi/ml) and [¹⁴C]inulin (8.5 Ci/mol) were obtained from the Commissariat à l'Energie Atomique (Gif sur Yvette, France). 2-Aminoisobutyric acid, *N*-methyaminoisobutyric acid, cycloleucine and albumin were from Sigma (St. Louis, MO, U.S.A.) and collagenase was from Boehringer (Meylan, France).

Animals and diets

Male Wistar rats weighing 250–270 g were fed on a high-carbohydrate diet (77% wheat starch and 13% casein, w/w) or a high-protein diet (90% casein). The other ingredients (4% corn oil, 5% salt

mixture and 1% vitamin) were in the same proportions for the two diets (of equal energy content). The salt and vitamin mixtures were purchased from UAR (Villemoisson sur Orge, France). Animals were fed from 09:00 to 17:00h. The lighting schedule was: light 21:00–09:00h, dark 09:00–21:00h.

Determination of alanine and leucine concentrations in liver and plasma

Fed rats were sampled 5h after the beginning of food intake. The rats were anaesthetized with sodium pentobarbital (40 mg/kg) and maintained at 37°C on a warming plate. After laparotomy, blood was withdrawn in heparinized syringes (1 ml) from either portal vein, then from the aorta or from hepatic vein (in the left hepatic lobe). Liver sampling and determination of alanine and leucine were performed as previously described (Rémésy *et al.*, 1978).

Preparation of isolated hepatocytes

Hepatocytes were isolated from fed rats after 10 days adaptation, at 14:00h, i.e. 5h after the onset of feeding. Collagenase dissociation was performed by the method of Berry & Friend (1969) as modified by Krebs *et al.* (1974). The integrity of the hepatocytes was estimated by cell-membrane refractoriness in phase-contrast microscopy and was about 90%. Incubation was performed in Krebs–Henseleit (1932) bicarbonate buffer containing 3% (w/v) albumin (pH 7.40).

Transport experiments

Amino acid transport was initiated in Eppendorf Microfuge tubes containing 2-amino[¹⁴C]isobutyrate or [¹⁴C]cycloleucine (0.1 μ Ci) by the addition of a 250 μ l hepatocyte suspension (about 3×10^6 cells/ml) in Krebs albumin buffer (Krebs *et al.*, 1974). The resulting solution was incubated at 37°C for 6 min with appropriate agitation. For measurement of the time course of 2-aminoisobutyrate uptake, 2 ml of hepatocyte suspension was incubated at 37°C with radioactive 2-aminoisobutyrate (1 μ Ci) over a period of 20 min in 20 ml

flasks, with agitation, and gassed with CO₂/O₂ (1:19). Samples (250 μ l) of hepatocyte suspension were taken at the required times. The reaction was stopped by addition of 1.0 ml of ice-cold Krebs albumin buffer to the microfuge tubes, which were immediately centrifuged for 5 s at about 1000 g. The supernatant was discarded and the cells were resuspended in 1.0 ml of chilled buffer. The same procedure was used to collect the cell pellets, which were dissolved in 100 μ l of formic acid before transfer to counting vials with 300 μ l of ethanol and 5 ml of scintillation liquid (Ready-Solv HP; Beckman, Gagny, France).

The residual extracellular radioactivity that was still associated with the cells after the washing procedure did not exceed 5% of the total uptake. By determination of the time course of 2-amino[¹⁴C]isobutyrate efflux at 0°C, it was shown that there was minor loss of radioactivity by the cells with this procedure (8% in 40 min); as the time of washing and isolation did not exceed 2 min, this factor is therefore not critical.

For calculation of 2-aminoisobutyrate distribution ratio between extra- and intra-cellular space, aqueous cell volume was determined by incubating hepatocytes with ³H₂O and [¹⁴C]inulin for 5 min at 37°C. In this last procedure, the incubation was stopped by centrifuging the cell suspension (800 μ l) in Eppendorf Microfuge tubes through 300 μ l of silicone oil (AR 200/AR 20, 1:3, v/v), (Wacker Chemie, Munich, Germany) layered above 200 μ l of 1.2 M-HClO₄.

Results

Alanine and leucine concentrations in liver and plasma from blood vessels supplying the liver

The arterial concentration of alanine was barely modified by increased dietary protein (0.77 and 0.92 mM with low- and high-protein diets respectively), in contrast with arterial leucine concentration (0.18 and 0.83 mM). As shown in Table 1, patterns of hepatic uptake of alanine and leucine were very different. Alanine was moderately removed by the liver of high-carbohydrate-fed rats

Table 1. Concentrations of alanine and leucine in liver and plasma in rats fed on low- or high-protein diets

It was assumed that portal vein and hepatic artery represent 70% and 30% of the afferent hepatic blood flow respectively. Afferent concentration is thus: 0.7 (portal-vein concn.) + 0.3 (aorta concn.) (Rémésy *et al.*, 1978). Values are means $\pm$ S.E.M. for ten rats.

	Diet ...	Concn. of alanine (mM)		Concn. of leucine (mM)	
		13% casein	90% casein	13% casein	90% casein
Afferent plasma		1.13 $\pm$ 0.06	2.07 $\pm$ 0.1	0.21 $\pm$ 0.01	1.23 $\pm$ 0.08
Liver		2.36 $\pm$ 0.15	1.43 $\pm$ 0.05	0.18 $\pm$ 0.01	0.68 $\pm$ 0.05
Hepatic vein		0.85 $\pm$ 0.05	0.59 $\pm$ 0.05	0.19 $\pm$ 0.01	0.84 $\pm$ 0.05

(uptake 25%). After adaptation to a high-protein diet, alanine uptake by liver was strongly increased; 72% of alanine reaching the liver was taken up. The hepatic uptake of leucine was extremely limited with a 13%-casein diet, whereas much more leucine was removed by the liver (32%) when leucine in the afferent plasma was dramatically increased by giving a high-protein diet.

Time course of 2-aminoisobutyrate uptake

Fig. 1(a) shows that 2-aminoisobutyrate uptake and distribution ratio (intracellular/extracellular concn.) at 0.1 mM concentration were higher in cells from rats fed on the higher-casein diet. 2-Aminoisobutyrate uptake by two batches of cells at high concentration of 2-aminoisobutyrate (50 mM) was of the same magnitude, with a slight stimulation in cells from rats on a 90%-casein diet (Fig. 1b).

Kinetic analysis of influx

2-Aminoisobutyrate uptake increased linearly with time, at least during 6 min, and thus the effect of substrate concentration could be determined after 6 min incubation. Fig. 2(a) shows the dependence of 2-aminoisobutyrate transport on substrate concentration; it appears that, over the entire concentration range, and particularly for the lowest concentration, there was a greater uptake of 2-aminoisobutyrate in hepatocytes from rats fed on a 90%-casein diet. Plots of rate against substrate concentration do not provide unequivocal information about the nature of the transport system implicated, because 2-aminoisobutyrate could be carried by two Na^+ -dependent systems (A and ASC). The plot of transport rate (v) against $v/[\text{substrate}]$ (Fig. 2b) indicates that 2-aminoisobutyrate transport was mediated by one or several carriers of similar affinity in hepatocytes from rats fed on a 13%-casein diet. In contrast, after adaptation of rats to a high-protein diet, liver parenchymal cells transported 2-aminoisobutyrate with carriers of various affinities. In Na^+ -free medium, uptake of 2-aminoisobutyrate by hepatocytes was not significantly different in rats adapted to either diet. The rate of uptake was linearly related to 2-aminoisobutyrate concentration; this observation agrees with the characteristics of a passive diffusion process.

Inhibition of 2-aminoisobutyrate uptake by L-alanine and L-serine

Both amino acids have been reported to be transported by the A and ASC systems (Christensen *et al.*, 1973), and these natural amino acids inhibit the Na^+ -dependent uptake of 2-aminoisobutyrate, which is also transported by both A and ASC systems. This allows us to determine the importance of the diffusion process for two batches of cells. At these

Fig. 1. Effect of dietary protein on the time course of 2-aminoisobutyrate uptake

Hepatocytes were isolated from rats fed on (○) 13%- or (●) 90%-casein diets, and were suspended in a medium containing 2-amino- ^{14}C isobutyrate at 0.1 mM (a) or 50 mM (b). The distribution ratios are the ratios of intracellular to extracellular concentrations. Intracellular water was similar for both nutritional conditions, in the range $3.5 \pm 0.2 \mu\text{l}/10^6$ cells. The values shown are the means of two determinations from three different hepatocyte suspensions.

concentrations of 2-aminoisobutyrate and inhibitors, this process contributed 20% and 40% of 2-aminoisobutyrate uptake by the cells from rats fed on 90%- or 13%-casein diets respectively (Fig. 3).

Characterization of induced transport system

Cycloleucine incorporation was measured in the absence of Na^+ for characterization of the L system.

Fig. 2. Effect of 2-aminoisobutyrate concentration on its transport rate

Hepatocytes isolated from rats fed on 13%- or 90%-casein diets were incubated for 6 min with 2-aminoisobutyrate concentrations in the range 0.1–30 mM. Choline was used as a substitute for Na⁺ when a Na⁺-free medium was required during transport assay. Fig. 2(a) represents rate of 2-aminoisobutyrate transport, and in Fig. 2(b) the data for Na⁺-dependent transport have been plotted according to the Woolf–Augustinson–Hofstee representation. The values shown are the means of two determinations from the same hepatocyte preparation. Circles represent experiments with Na⁺ (○, 13%-casein diet; ●, 90%-casein diet) and squares experiments without Na⁺ (□, 13%-casein diet; ■, 90%-casein diet).

No change in this last system was detected with the high-protein diet (Table 2).

To verify the emergence of the A system in hepatocytes from rats fed on 90%-casein diet,

Fig. 3. Inhibition by alanine and serine of 2-aminoisobutyrate influx in hepatocytes from rats fed on (○) 13%- or (●) 90%-casein diets

The initial rate of 2-amino[¹⁴C]isobutyrate uptake at 0.1 mM was measured with or without increasing concentrations of unlabelled L-alanine and L-serine. We have verified that the 2-aminoisobutyrate transport not inhibited by alanine and serine corresponds to the Na⁺-insensitive transport component. For this experiment, the incubation time was shortened to 4 min. During this time and with the cell concentration used (2 × 10⁶/ml), no significant changes in alanine and serine concentrations were observed at amino acid concentrations in excess of 1 mM; thus metabolic inhibitors were not used. Results are expressed as percentage of the maximal rate of 2-amino[¹⁴C]isobutyrate uptake measured in the absence of alanine and serine. Each value is the mean for two assays from two separate hepatocyte preparations. The hatched and stippled areas represent the non-inhibited aminoisobutyrate transport that was ascribed to passive diffusion for 13%- and 90%-casein diets respectively.

2-aminoisobutyrate incorporation was studied in the presence of *N*-methylaminoisobutyrate, which is a competitive inhibitor of the A system. There was no difference in 2-aminoisobutyrate uptake by cells

Table 2. Comparative changes in the activity of the A, L and ASC systems after adaptation to a high-protein diet: effects of dibutyryl cyclic AMP

The various systems were characterized as follows: L-system transporting cycloleucine in Na⁺-free medium; passive diffusion of 2-aminoisobutyrate in Na⁺-free medium; total transport in Na⁺ medium; inhibition of A system by *N*-methylisobutyrate. The effect of dibutyryl cyclic AMP on aminoisobutyrate transport was studied after 2 h incubation of hepatocytes in a Krebs albumin/bicarbonate buffer containing 0.1 mM-dibutyryl cyclic AMP; 2 h incubation did not affect 2-aminoisobutyrate transport by the control hepatocytes. Results are the mean transport rates $\pm$ S.E.M. for five determinations from two batches of hepatocytes.

Diet	Rate of transport of ¹⁴ C-labelled amino acid analogue (nmol/min per 10 ⁸ cells)				
	Choline medium		Na ⁺ medium		
	0.2 mM-cycloleucine	0.2 mM-2-aminoisobutyrate	0.2 mM-2-aminoisobutyrate	0.2 mM-2-aminoisobutyrate + 20 mM- <i>N</i> -methyl-2-aminoisobutyrate	0.2 mM-2-aminoisobutyrate + 0.1 mM-cyclic AMP
13% Casein	13.0 $\pm$ 1.2	2.0 $\pm$ 0.4	6.6 $\pm$ 0.6	5.2 $\pm$ 1.0	13.0 $\pm$ 1.0
90% Casein	11.6 $\pm$ 0.6	1.8 $\pm$ 0.4	10.6 $\pm$ 0.8	5.8 $\pm$ 0.8	32.0 $\pm$ 0.8

from the two groups of rats in the absence of Na⁺ or in the presence of Na⁺ and 20 mM-*N*-methylaminoisobutyrate. The emergence of the A system thus accounted for the increase in 2-aminoisobutyrate incorporation in rats fed on the high-protein diet. These changes were apparently mimicked by previous incubation with dibutyryl cyclic AMP, and the response to dibutyryl cyclic AMP was markedly higher after adaptation to the high-protein diet. (Table 2).

Discussion

In rats fed on high-carbohydrate diets, alanine was taken up by the liver against a concentration gradient, but uptake was relatively low, concomitant with digestive supply (Rémésy & Demigné, 1982). With high-protein diets, favourable concentration gradients were established for alanine uptake by the large supply from both the digestive tract and extrahepatic tissue metabolism and by the decrease in intracellular alanine, owing to the high activity of alanine aminotransferase (Kaplan & Pitot, 1970).

Apparently accelerated transport could easily be explained by an increase in the alanine concentration of afferent blood from about 1 to 2 mM, since the transport K_m for alanine is about 4 mM (Joseph *et al.*, 1978; Sips *et al.*, 1980). In fact alanine transport is certainly competitively inhibited by the increased concentration of several amino acids in the portal vein. Thus accelerated transport may result from induction or activation of various transport systems. The fact that intrahepatic alanine is lower in high-protein-fed rats than in low-protein-fed rats, despite the increased alanine supply, indicates that accelerated alanine metabolism must be more important than accelerated transport. With standard diets it is possible that transport is no longer limiting

for metabolism of alanine in excess of 1 mM when the activity of alanine aminotransferase (EC 2.6.1.2) is normal. But in high-protein-fed rats, the activity of the aminotransferase is about 6 times that in 13%-casein-fed rats (results not shown), and thus the threshold of blood alanine concentration for which the transport is limiting is certainly higher.

Leucine uptake was only observed in the presence of a favourable concentration gradient with high-protein diets, and in this case we have not observed any change in the activity of the L system. Various mechanisms could be invoked in explanation: adaptation of enzymes of leucine catabolism, such as leucine aminotransferase (EC 2.6.1.6) (Krebs, 1972) and 4-methyl-2-oxopentanoate dehydrogenase (EC 1.2.4.4) (Wohlhueter & Harper, 1970); increased transamination related to accumulation of leucine in the liver; increased protein synthesis.

The question arises of the participation of transport processes in the regulation of amino acid metabolism and interorgan relationships. The use of non-metabolizable analogues has been found suitable for investigating this problem, although the nature and the diversity of natural amino acid carriers (Joseph *et al.*, 1978; Kilberg *et al.*, 1980) are not necessarily reflected by these particular amino acids. Most amino acids are more efficiently taken up by the liver with high-protein diets (Rémésy *et al.*, 1978); it could therefore be interesting to assess if stimulation of the A system alone accounts for this phenomenon. In rats on high-carbohydrate diets, the plot of v against v/s was linear ($r = 0.80$) for the carrier system that could be identified as the ASC system, since the K_m was higher than 30 mM and the V_{max} estimated as 28 nmol/min per 10⁶ cells. In fact, among various Na⁺-dependent systems, the A system alone appeared responsive to dietary protein and accounted for the higher capacity for

2-aminoisobutyrate transport by hepatocytes; this view was supported by the fact that neither the L system (cycloleucine-transporting) nor the ASC system exhibited any substantial change. Identification of factors responsible for this adaptation is complex, as various hormonal influences for the induction of the A system have been reported: glucocorticoids, adrenaline (Canivet *et al.*, 1980), glucagon and cyclic AMP (Le Cam & Freychet, 1976). Glucagon, which is enhanced in rats fed on high-protein diets (Eisenstein & Strack, 1978), could be a candidate for stimulating amino acid transport, through cyclic AMP (Tews *et al.*, 1975). In fact, glucagon could induce a biphasic stimulation of amino acid uptake, the first phase being dependent on cell-membrane polarization and the second phase on protein synthesis (Edmondson & Lumeng, 1980). Moreover, cyclic AMP may also stimulate alanine uptake by an effect on intracellular metabolism (McGivan *et al.*, 1981). The present results show that dibutyl cyclic AMP may increase 2-aminoisobutyrate transport probably by the induction of the A system, as suggested by Le Cam & Freychet (1976) and Fehlmann *et al.* (1979). This response was more marked after adaptation to a high-protein diet and was particularly striking if the basal uptake of 2-aminoisobutyrate is considered. However, the origin of this difference is still unknown: higher rate of release of cyclic AMP from dibutyl cyclic AMP, or increase in the production of mRNA, or increase in the rate of protein synthesis. In support of our results, Tews & Harper (1976) have also reported that liver slices from rats adapted to high-carbohydrate diets were less responsive to glucagon or to cyclic AMP; however, insulin probably played a key role with such diets. In fact, the mechanisms of induction of transport systems are still speculative even if synthesis of protein seems required (Canivet *et al.*, 1980), for production of either new carrier units and/or regulatory proteins.

The present work suggests that the emergence of the A system takes place in fed rats after adaptation to a high-protein diet. The same phenomenon occurs in starvation, when transport processes and the low alanine concentration are certainly rate-limiting for metabolism. With high-protein diets, accelerated metabolism seems decisive, but more detailed studies are necessary for assessment of the limiting character of transport processes.

We gratefully acknowledge the technical assistance of Catherine Besson and the secretarial assistance of Françoise Thomas.

References

- Berry, M. N. & Friend, D. S. (1969) *J. Cell Biol.* **43**, 506–520
- Canivet, B., Fehlmann, M. & Freychet, P. (1980) *Mol. Cell. Endocrinol.* **19**, 253–261
- Christensen, H. N., de Cespedes, C., Handlogten, M. E. & Ronquist, G. (1973) *Biochim. Biophys. Acta* **300**, 487–522
- Edmondson, J. W. & Lumeng, L. (1980) *Biochem. Biophys. Res. Commun.* **96**, 61–68
- Eisenstein, A. B. & Strack, I. (1978) *Proc. Soc. Exp. Biol. Med.* **158**, 578–581
- Fehlmann, M., Le Cam, A. & Freychet, P. (1979) *J. Biol. Chem.* **254**, 10431–10437
- Guidotti, G. G., Borghetti, A. F. & Gazzola, G. C. (1978) *Biochim. Biophys. Acta* **515**, 329–366
- Joseph, S. K., Bradford, N. M. & McGivan, J. D. (1978) *Biochem. J.* **176**, 827–836
- Kaplan, J. H. & Pitot, H. C. (1970) in *Mammalian Protein Metabolism* (Munro, H. N., ed.), pp. 388–436, Academic Press, London and New York
- Kilberg, M. S., Handlogten, M. E. & Christensen, H. N. (1980) *J. Biol. Chem.* **255**, 4011–4019
- Krebs, H. A. (1972) *Adv. Enzyme Regul.* **10**, 397–420
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–36
- Krebs, H. A., Cornell, N. W., Lund, P. & Hems, R. (1974) in *Regulation of Hepatic Metabolism* (Lundquist, F. & Tystrup, N., eds.), pp. 726–750, Munksgaard, Copenhagen
- Le Cam, A. & Freychet, P. (1976) *Biochem. Biophys. Res. Commun.* **72**, 893–901
- Le Cam, A. & Freychet, P. (1977) *J. Biol. Chem.* **252**, 148–156
- McGivan, J. D., Ramsell, J. C. & Lacey, J. H. (1981) *Biochim. Biophys. Acta* **644**, 295–304
- Rémésy, C. & Demigné, C. (1982) *J. Nutr.* **112**, 60–69
- Rémésy, C., Demigné, C. & Aufrère, J. (1978) *Biochem. J.* **170**, 321–329
- Sips, H. J., Groen, A. K. & Tager, J. M. (1980) *FEBS Lett.* **119**, 271–274
- Tews, J. K. & Harper, A. E. (1976) *J. Nutr.* **106**, 1497–1506
- Tews, J. K., Colosi, N. W. & Harper, A. E. (1975) *Am. J. Physiol.* **228**, 1606–1614
- Wohlhueter, R. M. & Harper, A. E. (1970) *J. Biol. Chem.* **245**, 2391–2401