Metabolic Evidence for Adaptation to a High Protein Diet in Rats

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ABSTRACT This study was designed to assess the effects of long-term adaptation to a high protein diet on energy intake, body weight gain, body composition and splanchnic metabolic indicators in rats. For this purpose, adult male Wistar rats were fed either a 50 g/100 g dry matter (DM) protein diet (P50 group) or a 14 g/100 g DM protein diet (P14 group) for 21 d. These two groups were compared with a P14 pair-fed (P14-pf) group that consumed the same daily energy as the P50 group. The energy intake of the P50 group was 16 ± 1% less than that of the P14 group ($P < 0.05$), and the P50 group had significantly lower body weight. The P50 group had significantly less adipose tissue compared with both P14 and P14-pf rats. The activities of the brush border membrane enzymes, neutral aminopeptidase and γ-glutamyl transferase, were significantly higher in the P50 group than in the P14 rats. Similarly, the activities of alanine aminotransferase, arginase and serine dehydratase were significantly higher in the liver of P50 rats compared with P14 rats. Both amino acid transporter system A and $X_{A,G-}$ activities, measured in freshly isolated hepatocytes, were significantly higher in the P50 group (8- and 1.5-fold, $P < 0.05$, respectively) compared with the P14 group. The 1.5-fold increase in the steady-state activity of $X_{A,G-}$ was accompanied by a doubling of EAAT2 mRNA, involved in the system $X_{A,G-}$. This study provides confirmation that specific biochemical and molecular adaptive processes of the splanchnic area are involved in the response to variations in the protein content of the diet. J. Nutr. 131: 91–98, 2001.

KEY WORDS: • *rats* • *high protein diet* • *liver* • *amino acid transporters* • *gene expression.*

A clear differentiation is yet to be made between the level of protein intake leading to health benefits and the tolerable upper level of intake (1,2). It has been shown that increasing the level of protein from 10 to 20–25% can favor lean body mass and reduce body fat in rats (3), but it is not clear whether a higher protein intake might further enhance the lean body mass (4). Moreover, transient anorexia and a reduction in body weight are often noted when the protein intake rises above 50–55% dry matter (DM),[2] suggesting a limit to the metabolic adaptive capacities of rats (5–9). In addition, pancreatic growth, hepatomegaly, nephromegaly, interference with calcium homeostasis and a reduction in sympathetic activity in brown adipose tissue have also been reported, mainly in cases of very high protein intake (10–13). Clearly, a precise understanding of the biochemical and molecular events involved in metabolic adaptations to variations in protein intake could be of considerable value.

One important metabolic adjustment to the dietary protein level is nitrogen excretion, which is dependent on protein intake and reflects precisely any increase in amino acid oxidation and subsequent nitrogen elimination (14). Tissues in the splanchnic area, i.e., the intestinal mucosa and liver, are particularly affected by these adaptations. The upper level of protein intake to which they can adapt likely represents the limit between metabolic adaptive adjustment and adverse effects (15). Indeed, the activities of the different gut and liver enzymes and transport systems increase markedly after an adaptation period, thereby increasing the capacity for amino acid catabolism (16–20). Activities of hepatic transport systems may be adapted to the portal load. Several distinct amino acid transport systems have been characterized in the liver, including the Na^+-dependent systems A, ASC and $X_{A,G-}$. A high protein diet enhances alanine exchange by system A (21), whereas the effects on systems ASC and $X_{A,G-}$ are unclear. ASC is involved in neutral amino acid transport; under baseline conditions, it ensures a large proportion of alanine uptake by the liver (22). Two different proteins may account for the ASC transport system, i.e., ASCT1 and ASCT2 (23). ASCT2 was not detected in the liver, suggesting that ASCT1 may be the principal subtype in the liver (24). According to the classification proposed by Palacin and coworkers (25), five isoforms of the glutamate transporter proteins (EAAT1 to 5) may be responsible for $X_{A,G-}$ transport activity. Two of these isoforms, EAAT2 and EAAT4, are strongly expressed in the liver as well (personal observation, data not shown).

The objective of this work was thus to assess the effects of long-term adaptation to a diet with a moderately high protein content (50 g/100 g DM) on energy intake, body weight gain,

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[2] Abbreviations used: ALT, alanine aminotransferase; AST, aspartate aminotransferase; DM, dry matter; MeAIB, methyl-amino-isobutyric acid; P14, rats fed the 14 g/100 g protein diet; P14-pf, rats fed the P14 diet pair-fed to the P50 group in energy; P50, rats fed the 50 g/100 g protein diet; RT-PCR, reverse transcriptase-polymerase chain reaction; WAT, white adipose tissue.

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body composition and intestinal and hepatic metabolic indicators in rats. Metabolic markers included intestinal brush border enzymes (neutral aminopeptidase, dipeptidyl aminopeptidase IV, γ-glutamyltransferase), liver transamination [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], liver gluconeogenesis (threonine-serine dehydratase), liver ureagenesis (arginase), the liver's ammonia scavenging system (glutamine synthase) and liver amino acid transport capacities (system A, system ASC, system $X_{A,G}$-) and expression (system $X_{A,G}$- and system ASC).

MATERIALS AND METHODS

Chemicals and biochemicals. Collagenase A (0.96 U/mg) was purchased from Boehringer Mannheim (Meylan, France). L-[2,3-^{3}H] alanine (1.92 TBq/mmol) was purchased from Sigma (La Verpillière, France) and L-[G-^{3}H] glutamic acid (1.81 TBq/mmol) from the Radiochemical Center (Amersham, Les Ulis, France). All other reagents were ordered from Sigma (La Verpillière, France).

Animals and diets. Adult male Wistar rats ($n = 40$) from Harlan (Gannat, France) weighing 200–210 g at the beginning of the experiment were housed individually in stainless steel wire cages at 22 ± 2°C, on a 12-h reverse light:dark cycle (0700–1900 h; lights on at 1900 h). All experimental procedures used during these experiments complied with the guidelines of the French National Animal Care Committee. Twenty-four rats were used for body weight, food intake and body carcass measurements and 16 for intestinal and liver metabolic studies because liver collagenase dissociation and body carcass composition were not possible concomitantly. Two diets were used. The P14 diet was an AIN-93M modified diet (26). In place of casein and cystine, this diet contained 140 g of total milk protein per kilogram of diet. The P50 diet was also an AIN-93M modified diet, containing 500 g of total milk protein per kilogram of diet. The addition of protein replaced an equivalent amount of sucrose and starch. The composition of the test diets is shown in **Table 1**.

Body weight, food intake and body carcass measurements. For the first week (prefeeding period, until d 0) 24 rats were adapted to the laboratory conditions. They were divided into three groups of eight rats matched for body weight and called P14, P14-pair fed (P14-pf) and P50. During this prefeeding period, the three groups had free access to the standard diet, P14. For the next 3 wk (experimental period, d 1–21), the P14 and P50 groups had free access to the P14 or P50 diets, respectively. The P14 pair-fed group received the same daily energy intake as the P50 group. Food intake and body weights were measured daily at 1700 h. All diets were moistened (water/powdered diet, 1:2) to prevent spillage. Water was available at all times. After 21 d and overnight food deprivation, the rats were weighed and killed using sodium pentobarbital (45 mg/kg body). Brown adipose tissue from the interscapular fat pad and four deposits of white adipose tissue (WAT), the epididymal, retroperitoneal, visceral and subcutaneous fat pads, were carefully removed and weighed. Liver, digestive tract, heart, kidneys and spleen were excised, washed free of gross blood in a NaCl 9 g/L solution and weighed. The stripped carcass was also weighed.

TABLE 1

Composition of the experimental diets P14 and P50[1]

	High protein diet (P50)	Normal protein diet (P14)
Metabolizable energy, *kJ/g*	15.8	14.9
	g/kg dry matter	
Total milk protein[2]	500	140
Cornstarch[3]	312.7	622.4
Sucrose[4]	50	100.3
Soybean oil[5]	40	40
AIN 93M mineral mix[6]	35	35
AIN 93V vitamin mix[6]	10	10
α-Cellulose[7]	50	50
Choline[6]	2.3	2.3

[1] All dietary components were purchased (sources given above) or prepared by the A.P.A.E. (Atelier de Preparation des Aliments Expérimentaux, French National Institute of Agronomic Research, INRA, Jouy en Josas, France).
[2] Nutrinov, Rennes, France.
[3] Cerestar, Haubourdin, France.
[4] Eurosucre, Paris, France.
[5] Bailly SA, Aulnay-sous-bois, France.
[6] ICN biochemicals, Cleveland, OH.
[7] Medias filtrants Durieux, Torcy, France.

Intestinal and hepatocyte experiments. After wk 1 of acclimatization, 16 rats were divided into two groups of eight rats also called P14 and P50. Rats consumed ad libitum the P14 and P50 diets, and were housed as previously described. After 21 d, these rats were anesthetized with an injection of pentobarbital (45 mg/kg body) for the isolation of hepatocytes and intestinal sampling.

Intestinal brush border homogenate preparation. Small intestines were removed during liver collagenase dissociation (see below), placed in an ice-cold 9 g/L NaCl solution and divided into two equal pieces (proximal and distal). After being washed twice with the same solution, the gut pieces were everted and scraped. The homogenates were stored immediately at −80°C. Enzymatic assays were conducted on the homogenates during the week after the sampling.

Preparation of isolated hepatocytes. Liver cells were isolated using the collagenase dissociation method described by Seglen with a few modifications. Livers were perfused in situ through the lower vena cava at a rate of 40 mL/min with a HEPES buffer (HEPES 10 mmol/L, NaCl 137 mmol/L, Na_2HPO_4, 0.7 mmol/L, pH 7.65, 37°C) (27). A slit was made in the portal vein to allow the free outflow of the perfusate. After 30 min, $CaCl_2$ (2.7 mmol/L) and collagenase (0.2 g/L) were added to the buffer and the perfusion was continued for another 15 min. The collagenase-digested liver was carefully removed and gently broken up in a Petri dish to disperse cells. The cell suspension was passed over a nylon mesh and washed free of collagenase with HEPES buffer. A Percoll continuous gradient (1 volume of cell suspension/2 volumes Percoll) in HEPES buffer was used to separate hepatocytes from nonparenchymal liver cells. Cell viability was ~90–95%, as estimated by trypan blue exclusion. Freshly isolated hepatocytes were used immediately for amino acid uptake measurements or frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

Enzyme assays. All determinations of enzyme activities were performed spectrophotometrically. Neutral aminopeptidase (EC 3. 4.11.11) and dipeptidyl aminopeptidase activities (EC 3.4.14.5) were assayed in the intestine using the technique described by Maroux et al. (28). Gut γ-glutamyl transferase (EC 2.3.2.2) activity was assayed using a kit (Sigma diagnostic kits, La Verpillière, France). Liver enzyme activities were measured on cell homogenates prepared by the sonication of 1×10^6 hepatocytes for 5 s. Alanine aminotransferase (EC 2.6.1.2) and AST (EC 2.6.1.1) assays were performed as described by Bergmeyer et al. (29). Serine-threonine dehydratase (EC 4.2.1.16) activity was measured using the method described by Friedemann and Heugen (30). Arginase (EC 3.5.3.1) activity was determined using the method described by Schimke (31). Glutamine synthase (EC 6.3.1.2) was assayed indirectly on the basis of the formation of L-γ-glutamylhydroxamic acid from glutamate and hydroxylamine. L-γ-Glutamylhydroxamate was assayed colorimetrically after reaction with $FeCl_3$ (32). The protein content was determined using the bicinchoninic acid method from Pierce (Rockford, IL) (33) with bovine serum albumin as a standard. Results were expressed as moles of product formed per minute per milligram of protein.

Transport experiments. Amino acid uptake was measured at 37°C in isolated rat hepatocytes using a rapid filtration method. Briefly, 100 μL of cell suspension containing 1×10^6 viable hepatocytes was preincubated for 2 min at 37°C. Uptake was initiated by adding 900 μL of transport buffer containing 10 μmol/L L-glutamate or 100 μmol/L L-alanine trace-labeled with 3.7 GBq/L of [^{3}H]-L-glutamate or [^{3}H]-L-alanine, respectively. After 2 min, the reaction

was stopped by the addition of 4 mL of ice-cold stop buffer (transport buffer devoid of radiolabeled amino acid), and rapid filtration through a membrane filter (Millipore HAWP, 0.45 μm, Les Ulis, France). The filters were washed twice with 4 mL of the ice-cold stop buffer and the amount of amino acid transported was measured by liquid scintillation counting. The composition of the transport buffer was as follows: HEPES 10, mmol/L; NaCl, 137 mmol/L; KCl, 5 mmol/L; $CaCl_2$, 2.8 mmol/L; $MgSO_4$, 1 mmol/L; Na_2HPO_4, 0.3 mmol/L; KH_2PO_4, 0.3 mmol/L; glucose, 10 mmol/L; and (aminooxy)acetic acid, 0.5 mmol/L, pH 7.6. A transamination inhibitor, (aminooxy)-acetic acid, was used to prevent the metabolism of alanine and glutamate. An Na^+-free transport buffer (prepared by replacing NaCl with choline chloride and omitting Na_2HPO_4) was used to determine the Na^+-independent uptake of glutamate or alanine. Na^+-dependent transport of L-glutamate or L-alanine was calculated as the difference between the transport measured in Na^+-containing and Na^+-free buffers. Specific inhibitors were also used to determine the contribution of individual transport systems to the uptake of alanine and glutamate. To determine the glutamate transport through the $X_{A,G}$- system, Na^+-dependent glutamate uptake occurring in the presence of a 100-fold excess of D-aspartate was subtracted from the uptake measured in the absence of D-aspartate. Similarly, the contribution of system A to L-alanine transport was calculated from the difference between Na^+-dependent alanine uptake and the uptake measured in the presence of a 100-fold excess of methyl-amino-isobutyric acid (MeAIB). Alanine remaining after Na^+-dependent uptake, i.e., the proportion unaffected by MeAIB, was assumed to represent the contribution of system ASC. Results are expressed as picomoles of amino acid transported per milligram of cell protein.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Freshly isolated hepatocytes were resuspended in a denaturing solution (guanidinium thiocyanate, 4 mol/L; sodium citrate, 25 mmol/L; sarcosyl, 5 g/L, pH 7). Total RNA was extracted according to the method described by Chomczynski and Sacchi (34). After RNA integrity had been confirmed by ethidium bromide staining, first strand cDNA was synthesized from 4 μg of total RNA using oligo(dT)12–18 as primers in the presence of Moloney murine leukemia virus reverse transcriptase (Life Technology, Cergy, France) for 1 h at 37°C. For PCR, 5 μL of each RT product was used (50 μL final reaction volume), with primers chosen from conserved parts of the coding regions of EAAT1, EAAT2, EAAT3, EAAT4 and ASCT1 (**Table 2**). Amplifications were performed using a MJ Research PTC-200 Thermocycler (Prolabo, Fontenay-sous-bois, France) for 35 cycles, which comprised denaturation (95°C, 45 s), annealing (56°C, 45 s), and extension (72°C, 1 min) with the AmpliTaq Gold DNA polymerase (Perkin Elmer, Villebon, France). To permit semiquantitative analysis, RT-PCR of the housekeeping gene β-actin was also amplified and used as an internal standard for the PCR. After amplification, 10 μL of each EAAT1 (384 bp), EAAT2 (914 bp), EAAT3 (539 bp), EAAT4 (479 bp) and ASCT1 (495 bp) PCR reaction product was mixed with 4 μL of the product of the β-actin (473 bp) PCR reaction, and the PCR products were separated by electrophoresis through a 2% agarose gel, stained with ethidium bromide and quantified using the Alpha Innotech digital imaging system (Alpha Innotech, San Leandro, CA). The PCR products were sequenced by Cybergene (Paris, France).

Statistical analysis. Data are given as means ± SEM Statistical analyses were performed using Student's *t* test for comparison of amino acid uptakes and enzyme activities between the P14 and the P50 groups. Food intake and body weight comparisons between groups were performed using Tukey's test after ANOVA had established significant differences among the groups. All analyses were performed using the SAS Statistical package (SAS/STAT version 6.12 for windows 95, SAS Institute, Cary, NC). Probability values < 0.05 were considered to indicate significant differences.

TABLE 2

Oligonucleotide primers designed for reverse transcriptase-polymerase chain reaction

Gene	Sequences
EAAT1	5′-CGCTGTCATTGTGGGTACAATCC-3′
	5′-GCTGCCCCCCAATCACACCCAT-3′
EAAT2	5′-GTGCCAACAATATGCCCAAGC-3′
	5′-CCTAGCAACCACTTCTAAGTCC-3′
EAAT3	5′-GGGAAGATCATAGAAGTTGAA-3′
	5′-TGAACCGGTCCAGGAGCCAGT-3′
EAAT4	5′-CCTACCGCCAGATCAAGTACTTC-3′
	5′-GGTGACATTTTCCAGGATGCT-3′
ASCT1	5′-GGGAAATACATCTTCGCATC-3′
	5′-GTGGTGGTCCGGTCCACAAT-3′
β-Actin	5′-TGGAATCCTGTGGCATCCATGAAA-3′
	5′-TAAAACGCAGCTCAGTAACAGTCCG-3′

RESULTS

Energy intake and growth rate. During the prefeeding period (until d 0) all groups fed the P14 diet had similar daily intake (360 ± 4 kJ/d) and growth rate (4.4 ± 0.2 g/d). During the experimental feeding period (d 1–21), the rats consuming the P50 diet had 16 ± 1% lower energy intakes and body weight than those consuming the P14 diet (**Fig. 1**). Energy intakes were 323 ± 7 and 387 ± 6 kJ/d, respectively ($P < 0.001$). The lower energy intake in the P50 group was due in part to a considerably lower energy intake on d 1 (−28%, $P < 0.001$). By the end of the experiment, the body weight gain was ~86.0 ± 5.3 g in the P50 group vs. 112.7 ± 4.3 g in the P14 group ($P < 0.01$). However, there were no differences in body weight gain between the P50 and P14-pf groups. Moreover, at the end of the experiment, energy efficiency did not differ among the three groups, with a mean energy efficiency value of 0.014 ± 0.001 g gain/kJ.

Body composition. Body weight was significantly lower in both the P50 and P14-pf groups compared with the P14 group, whereas no difference was observed between the P50 and P14-pf rats (**Table 3**). The stripped carcass weight was significantly lower in the P14-pf group compared with the P14 group, whereas the P14 and the P50 groups did not differ. In addition, the stripped carcass/body weight ratio was significantly higher in the P50 group compared with the P14 group, whereas no difference was observed between the P14 and P14-pf groups. Comparisons of organ weights between the P14 and P14-pf groups indicated a lower weight for the liver, spleen and heart in the P14-pf group. In contrast, when the P50 group was compared with both the P14 and the P14-pf groups, liver, spleen and heart weights did not differ but kidney weight was greater in P50 rats. The P50 group had less adipose tissue than either the P14 and or the P14-pf rats. Adipose tissue weight was significantly lower in the P14-pf group than in the P14 group as well, but the effect was less marked than in the P50 group. Similar differences were also observed in the WAT/stripped carcass ratio.

Activities of intestinal and liver enzymes and hepatocyte amino acid transport systems. The activities of the intestinal brush border membrane enzymes, neutral aminopeptidase and γ-glutamyl-transferase, were all significantly greater in the P50 group than in the P14 group (**Table 4**). Similarly, in the liver, the activities of ALT, arginase and serine dehydratase were significantly higher in the P50 group, whereas AST and glutamine synthase activities were not affected by diet. Moreover, in rat liver cells obtained from rats fed the high protein diet for 21 d, a twofold increase in total alanine uptake was observed (**Table 5**). With both diets, the accumulated level of alanine was significantly reduced when choline chloride was substituted for sodium chloride in the transport buffer (sodium-independent transport). Furthermore, in the presence of

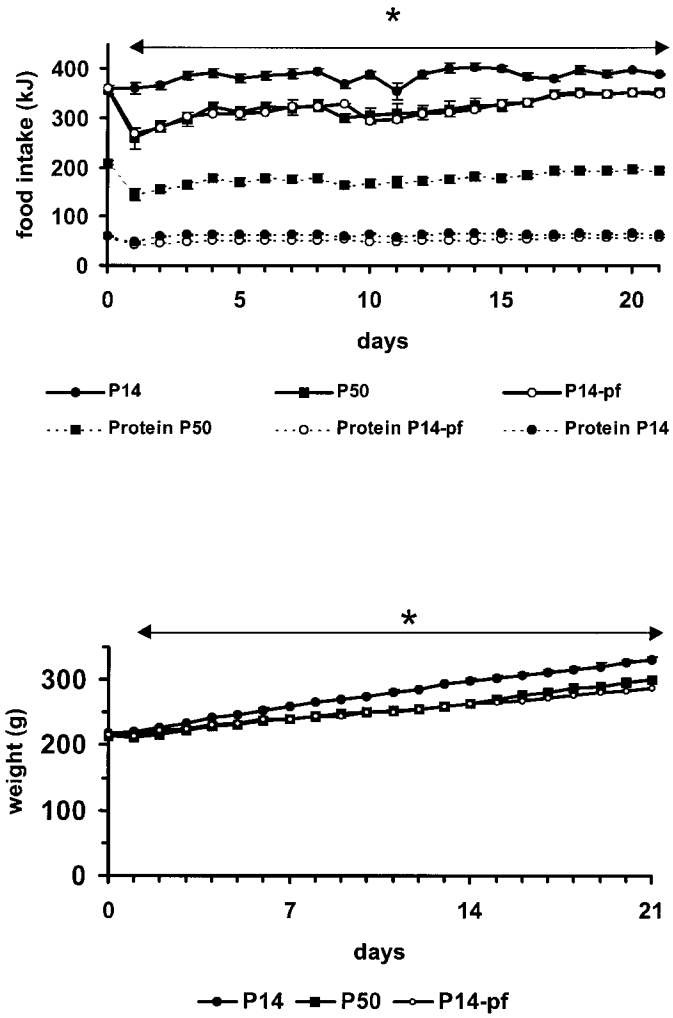

FIGURE 1 Daily total and protein energy intakes (*upper panel*) and body weight (*lower panel*) of rats fed either a 14 g/100 g protein diet (P14), a 50 g/100 g protein diet (P50) or a 14 g/100 g protein diet pair-fed to the P50 group in energy (P14-pf) for 21 d. Values are means ± SEM, $n = 24$. *P50 value is different from P14, $P < 0.05$. From d 1 to 21, the P14 rats consumed more energy than the P50 and the P14-pf rats. Additionally, during this period, the P50 rats consumed more protein energy than the rats from the other groups. There was no difference between the body weights of the P14-pf rats and P50 rats.

MeAIB, a reduction in alanine transport was observed. Under these conditions, system A activity (characterized by the difference in alanine accumulation in the presence or absence of MeAIB, at a 100-fold excess level) was approximately eight-fold greater in the P50 group than in the P14 group. By contrast, ASC activity (characterized by the Na^+-dependent alanine transport minus system A activity) was not significantly affected by the dietary protein level. Global glutamate uptake was higher in liver cells of P50 rats compared with those of P14 rats. In both diet groups, the addition of D-aspartate to the transport buffer decreased glutamate entry into the cells. The dietary protein level also influenced $X_{A,G}$- activity, which was higher in the P50 diet group.

Expression of EAAT2, EAAT4 and ASCT1 in hepatocytes. To investigate the effect of a high protein diet on the expression of $X_{A,G}$- isoforms further, semiquantitative RT-PCR assays were performed. EAAT1 and EAAT3 were present, but were too poorly expressed to be quantified accurately (data not shown). By contrast, EAAT2 and EAAT4 were strongly expressed. A doubling of EAAT2 mRNA was noted in rats that consumed the high protein diet for 3 wk compared with the P14 group, as shown by the analysis of its level of expression (**Fig. 2**). Values represent the ratio of EAAT2 mRNA to β-actin mRNA. In contrast, the expression of EAAT4 did not differ in the two groups (EAAT4/β-actin ratio of 67.6 ± 3.6 vs. 71.8 ± 10.7). For ASCT1, a zwitterionic amino acid transporter responsible for ASC activity in the liver, the high protein diet had no effect on the mRNA expression of this gene (ASCT1/β-actin ratio of 87.5 ± 10.6 vs. 90.5 ± 8.6).

DISCUSSION

Neither beneficial nor adverse health consequences of long-term ingestion of high protein diets have been elucidated. The difference between a situation requiring a metabolic adaptation and one with adverse health effects has not been determined. Adaptation is defined as a purposive response to a new feeding circumstance that results in a functional state that is better suited to the changed situation (35). This study showed that introduction of a moderately high protein diet produced a depression in energy intake, a reduction in the fat mass of rats and a higher ratio of lean to fat mass. Metabolic markers involved in liver amino acid catabolism were enhanced; among these, the amino acid transport systems A and $X_{A,G}$- appeared to be important targets for adaptation to protein intake. In this study, Wistar rats consuming the P50 diet had lower energy intakes than the control P14 group for the 21 d of the experiment (Fig. 1). The initial reduction in energy intake occurred rapidly, i.e., in a few hours, as previously described (9). Depending on the rat strain and the experimental conditions, rats were shown either to return gradually to the same level of intake as the control group after several days, or to maintain a reduced energy intake (6,17). The reasons for this long-term depression in energy intake are still poorly understood, but may be related to the satiating effect of proteins (36,37).

To discriminate between the effect of depression in energy intake and the effect of the high protein content of the diet, a pair-fed group (14 g/100 g) was included in the experiment. Interestingly, we noted different effects on body composition in the P50 and P14-pf groups, suggesting a specific effect of the dietary protein concentration. The principal effect on body composition in the P14-pf group (having a reduction in energy intake without any changes in the macronutrient composition of the diet) was a decrease in body weight associated with an overall decrease in the mass of tissues and organs, including lean body mass, adipose tissue, liver, spleen and heart (Table 3). In contrast, the reduction in body weight observed in the P50 group was associated mainly with a reduction in fat mass compared with both the P14 and the P14-pf groups, without any significant effects on lean body mass. This resulted in an increase in the ratio between lean and fat mass. Both the anorexic effect and the reduction in fat mass in response to a high protein diet could be related to the specific orientation of energy metabolism, with amino acids as the principal energy substrate. Indeed, the diversion of amino acids to catabolic pathways and gluconeogenesis is generally associated with a higher thermogenic effect of the diet (38). The precise mechanisms involved in such diversion, and the role of amino acid transport systems in directing amino acids specifically into the various metabolic pathways require further elucidation.

Adaptation to a high protein diet has been correlated with an increase in the activity of enzymes involved in protein digestion and in splanchnic nitrogen metabolism (9,17).

TABLE 3

Carcass composition of rats fed either a 14 g/100 g protein diet (P14), a 50 g/100 g protein diet (P50) or the P14 diet pair-fed to the P50 group in energy (P14-pf)[1,2]

	P14	P14-pf	P50
		g	
Body weight	330.0 ± 5.1[a]	292.9 ± 3.8[b]	299.0 ± 7.6[b]
Stripped carcass	166.1 ± 1.4[a]	150.43 ± 2.1[b]	157.1 ± 6.1[ab]
Stripped carcass/BW, *g/g*	0.57 ± 0.1[a]	0.58 ± 0.01[ab]	0.61 ± 0.02[b]
Liver	9.61 ± 0.32[a]	8.38 ± 0.10[b]	8.87 ± 0.44[ab]
Kidney	2.19 ± 0.07[a]	2.08 ± 0.06[a]	2.44 ± 0.06[b]
Spleen	0.84 ± 0.02[a]	0.72 ± 0.02[b]	0.79 ± 0.04[ab]
Heart	0.88 ± 0.02[a]	0.78 ± 0.01[b]	0.79 ± 0.04[ab]
Digestive tract	14.61 ± 0.67	13.30 ± 0.52	14.85 ± 0.53
Total WAT	32.36 ± 1.82[a]	21.85 ± 1.92[b]	16.60 ± 1.66[c]
Epididymal WAT	6.48 ± 0.35[a]	4.60 ± 0.37[b]	3.59 ± 0.25[c]
Retroperitoneal WAT	8.13 ± 0.47[a]	5.17 ± 0.58[b]	4.20 ± 0.62[b]
Subcutaneous WAT	11.63 ± 0.85[a]	7.78 ± 0.75[b]	4.89 ± 0.45[c]
Mesenteric WAT	6.13 ± 0.39[a]	4.29 ± 0.32[b]	3.92 ± 0.48[b]
BAT	0.62 ± 0.04[a]	0.55 ± 0.03[a]	0.43 ± 0.05[b]
WAT/stripped carcass, *g/g*	0.22 ± 0.01[a]	0.16 ± 0.01[b]	0.12 ± 0.01[c]

[1] Values are means ± SEM, $n = 24$. Values in a row without a common letter differ, $P < 0.05$.
[2] BW, body weight; BAT, brown adipose tissue; WAT, white adipose tissue.

Moreover, splanchnic amino acid catabolism appears to be a major adaptive response to an increase in the protein content of the diet (39,40). The high protein diet significantly enhanced the levels of intestinal peptidases (Table 4), consistent with previous findings that intestinal enzymes and peptide and amino acid transporters adapt to diets with different protein contents (16). These results also indicated that different pathways involved in liver amino acid metabolism were activated, including amino acid transport, transaminations, and the capacities for gluconeogenesis and ureagenesis. Indeed, increasing the protein intake produced more postprandial portal amino acid flow, and the liver has been shown to play a prominent role in the first-pass metabolism of these amino acids. It has become increasingly apparent that the activities of membrane proteins catalyzing the selective transport of amino acids play a major mechanistic role in the regulation of hepatic physiology, particularly when the amino acid flow is increased. These results strongly suggest that in periportal hepatocytes, which represent the largest liver parenchymal cell population, the response to a high protein diet is driven by the modulations of system A activity and subsequent catabolic enzyme systems. In perivenous hepatocytes, activation of system $X_{A,G}^-$ may play a scavenging role by increasing glutamate uptake and the subsequent transfer of ammonia to glutamate through glutamine synthase activity (41).

Our results clearly demonstrated a dramatic activation of system A in the liver by a high protein diet without major nutritional regulation of system ASC. The recent identification of the protein rATA2 responsible for the system A amino acid transport activity will allow further investigation of the cellular mechanisms involved in its up-regulation by high protein diets (42). The expression of ASCT1 mRNA, mea-

TABLE 4

Intestinal and liver enzyme activities in rats fed either a 14 g/100 g protein diet (P14) or a 50 g/100 g protein diet (P50)[1]

	P14	P50
Intestinal enzymatic activities, *mU/mg protein*		
Neutral aminopeptidase		
Proximal	876 ± 154	1025 ± 171*
Distal	912 ± 104	1104 ± 101*
Dipeptidyl aminopeptidase IV		
Proximal	322 ± 55	365 ± 39*
Distal	141 ± 72	639 ± 109*
γ-Glutamyltransferase		
Proximal	342 ± 90	459 ± 81*
Distal	295 ± 53	404 ± 47*
Hepatic enzymatic activities		
Alanine aminotransferase, *mmol NADH/(min · mg protein)*	224.3 ± 16.8	512.3 ± 30.9*
Aspartate aminotransferase, *mmol NADH/(min · mg protein)*	1048.7 ± 249.8	1352.7 ± 273.3
Arginase, *μmol urea/(min · mg protein)*	2.85 ± 0.10	3.56 ± 0.3*
Serine threonine dehydratase, *μmol oxobutyrate/(min · mg protein)*	83.11 ± 4.5	125.7 ± 4.5*
Glutamine synthase, *μmol γ-glutamyl hydroxamate/(min · mg protein)*	3.6 ± 0.3	4.3 ± 0.2

[1] Values are means ± SEM, $n = 16$. * Different from P14, $P < 0.05$.

TABLE 5

Glutamic acid and alanine accumulation in hepatocytes isolated from rats fed either a 14 g/100 g protein diet (P14) or a 50 g/100 g protein diet (P50)[1]

Uptake	P14	P50
	pmol/(min · mg protein)	
Alanine, *100 μmol/L*		
Total	54.4 ± 3.9	152.8 ± 17.4*
Na^+-independent	29.1 ± 2.3	37.3 ± 5.05
Na^+-dependent	25.3 ± 3.7	101.1 ± 12.2*
MeAIB, *10 mmol/L*	47.0 ± 4.1	82.9 ± 6.3*
Glutamate, *10 μmol/L*		
Total	6.3 ± 0.6	13.0 ± 1.0*
D-Aspartate, *1 mmol/L*	4.5 ± 0.7	8.0 ± 0.7*
Transport system activities		
System A	7.4 ± 1.9	69.4 ± 10.2*
System ASC	17.9 ± 3.1	32.6 ± 9.9
System X_{AG^-}	1.8 ± 0.4	4.9 ± 1.5*

[1] Values are means ± SEM, $n = 16$. * Different from P14, $P < 0.05$.

sured using semiquantitative RT-PCR, was not affected by the dietary protein level, and ASCT1 protein expression parallels system ASC activity. System A components in hepatocytes have been reported to be elicited in catabolic states such as chronic starvation (43), a very high protein diet (~90% casein) (44) and burn injury (22) or in hyperinsulininemic rats (45). The activation of system A in hepatocytes was associated with an increase in the activities of enzymes involved in amino acid catabolism, including ALT, arginase and serine dehydratase (Table 4). Such activation ensures the efficient uptake and subsequent catabolism of amino acids by periportal hepatocytes, thus preventing a rise in their peripheral concentration with a high protein diet. The additional increase in ALT activity drives certain amino acids toward catabolic pathways. Indeed, liver arginase activity was clearly higher in rats fed the high protein diet, showing that amino acids in excess of immediate requirements for synthesis were deaminated, and their nitrogen oriented towards the urea cycle. As already documented, increased amino acid catabolism and subsequent urea excretion are associated with an increase in kidney size (Table 3), and these have been related to a higher glomerular filtration rate (46). In addition, rat liver serine dehydratase, which catalyzes the pyridoxal phosphate–dependent deamination of L-serine or L-threonine to produce pyruvate or α-ketobutyrate, is regarded as a gluconeogenic enzyme. To date, it has been shown that serine dehydratase is increased dramatically under conditions of gluconeogenesis, such as starvation, diabetes mellitus or after the consumption of excess dietary protein (90% casein) (47). Even if the dietary protein concentration is increased only slightly (2-fold), the high protein diet induced dramatic stimulation (~50%) of serine dehydratase activity (Table 4). Because total oxidation of any substrate is impossible, it has been suggested that the principal pathway of amino acid carbon metabolism in the liver of rats fed a high protein diet is conversion to glucose (38).

Interestingly, transport across the liver sinusoidal membrane is recognized as an important mechanism controlling hepatic flux. In particular, the transport of glutamate, as a precursor of glutamine, is much higher in perivenous than in periportal hepatocytes, suggesting a major role in ammonia detoxification (48). Liver glutamate transmembrane delivery is thus crucial in the event of a dietary protein excess because when surplus ammonia escapes liver urea synthesis, glutamine may be synthesized from glutamate through glutamine synthase, acting as a scavenger for ammonia (49). Indeed, it has been shown previously that glutamate transport is enhanced when catabolic hormones (such as corticosteroid and glucagon) are secreted (50). In our study, we found that a high protein diet increased X_{A,G^-} activity without modifying glutamine-synthase activity (Tables 4 and 5). This finding is consistent with previous observations made in catabolic states such as major burn injury, where no appreciable increase in glutamine synthase expression was observed in the liver (51). Liver EAAT 2 and 4 subtypes participate mainly in X_{A,G^-} activity in this tissue (personal observation, data not shown). To improve our understanding of the mechanisms involved in the nutritional regulation of EAAT2 and EAAT4 gene expression, we investigated the effects of dietary protein on the abundance of EAAT 2 and EAAT4 mRNA to determine whether an increase in X_{A,G^-} activity was associated with a similar increase in EAAT mRNA. The 1.5-fold increase in steady-state X_{A,G^-} activity was accompanied by a doubling increase in EAAT2 mRNA (Fig. 2). The comparable magnitude of the change in activity and mRNA suggested that dietary protein may regulate X_{A,G^-} at the transcriptional level. However, we did not investigate the effect of dietary protein on EAAT protein level. This capacity for the adaptation in X_{A,G^-} activity in various catabolic states was consistent with observations made by Farfournoux and co-workers (52). However, the mechanism(s) by which dietary protein regulate(s) EAAT gene expression remain to be determined. The signals responsible for long-term regulation of X_{A,G^-} may be similar to those identified for other amino acid transporters. Insulin, glucagon, glucocorticosteroids and thyroid hormones have been identified as factors modulating amino acid transport and catabolism (21,45).

The results of this study suggest that rats fed a moderately

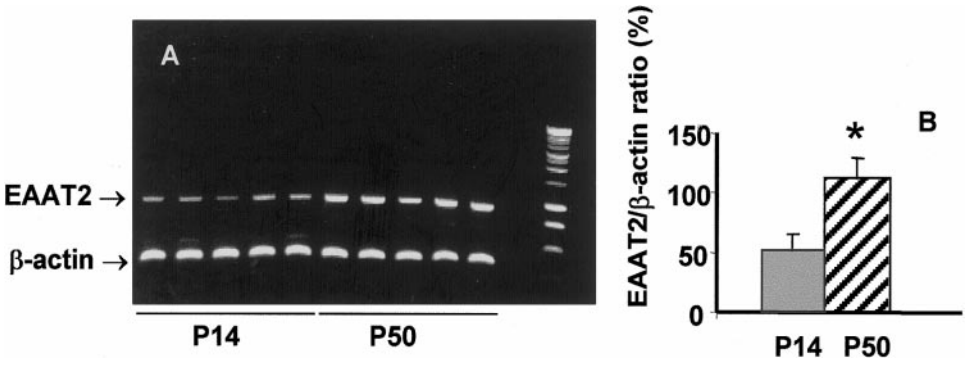

FIGURE 2 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of liver EAAT2 amino acid transporter mRNA from rats fed 14 (P14) and 50 g/100 g (P50) protein diets. PCR products were analyzed on a 20% agarose gel stained with ethidium bromide. The β-actin gene was used as an internal standard for RT-PCR reaction. (*A*) Quantification of the PCR product was achieved by densitometric analysis and the amount of the EAAT2 PCR fragment was expressed relative to the β-actin fragment. (*B*) Results are means ± SEM, $n = 10$. *Significantly different from P14, $P < 0.05$.

high protein diet successfully adapt to the dietary protein concentration within 2 wk because their body weights, energy intake and energy efficiency were similar to those of the pair-fed controls. The results provide confirmation that specific adaptive processes are involved in the response to variations in the protein content of the diet. In the splanchnic area, and more particularly in the liver, amino acid transport systems, including both system A and system $X_{A,G}$- by way of EAAT2, are controlled by the dietary protein concentration. Enhanced transmembrane amino acid delivery is associated with increased activities of catabolic pathways, such as the urea cycle and gluconeogenesis. Further studies are required to establish the nature of the transcriptional control of the EAAT2 gene, particularly by hormones. In the future, identification of the proteins involved in the regulation of amino acid transporters and enzymes of the amino acid metabolism will provide information at the molecular level about the basic mechanisms controlling body nitrogen and amino acid homeostasis and their relationship with energy metabolism and the control of body weight. Further studies are also required for precise determination of the physiologic tolerable upper limits for dietary proteins.

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