SPLANCHNIC SEQUESTRATION OF AMINO ACIDS IN AGED RATS: 
IN VIVO AND EX VIVO EXPERIMENTS USING A MODEL OF 
ISOLATED PERFUSED LIVER

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Running head

Liver role in splanchnic sequestration of amino acids

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ABSTRACT

Background: Splanchnic sequestration of amino acids (SSAA) is a process observed during aging that leads to decreased peripheral amino acid (AA) availability. The mechanisms underlying SSAA remain unknown. The aim of this study was to determine whether a high-protein diet could increase nitrogen retention in aged rats by saturating SSAA, and whether SSAA could be explained by dysregulation of hepatic nitrogen metabolism per se.

Materials and methods: Adult and aged male Sprague-Dawley rats were housed in individual metabolic cages and fed a normal-protein (17% protein) or a high-protein diet (27%) for two weeks. Nitrogen balance (NB) was calculated daily. On Day 14, livers were isolated and perfused (IPL) for 90 min to study AA and urea fluxes.

Results: NB was lower in aged rats fed a normal-protein diet than in adults, but a high-protein diet restored it to adult levels. IPL from aged rats showed decreased urea production and arginine uptake, together with a release of alanine (versus uptake in adult rats) and a hepatic accumulation of alanine.

Discussion: The in vivo data suggest that SSAA is a saturable process that responds to an increase in dietary protein content. The hepatic metabolism of AA in aged rats is greatly modified, and urea production decreases. This result refutes the hypothesis that SSAA is associated with an increase in AA disposal via urea production.
KEYWORDS

Aging, nitrogen metabolism, isolated perfused liver, amino acids, high-protein diet
INTRODUCTION

Sarcopenia is the universal, involuntary decline in lean body mass that occurs with aging (30). The resulting muscle weakness and function loss increase the incidence of falls in the elderly, whence an increased occurrence of fractures and a decline in quality of life (31). Understanding the process of sarcopenia could allow new therapeutic strategies designed to prevent the loss of muscle mass and function, thus preserving the quality of life of elderly people and decreasing morbidity and mortality. The context of aging populations in western countries makes this issue a major public health concern.

The underlying mechanism of sarcopenia is clearly multifactorial, and altered hormone secretion (54), undernutrition and lack of adequate physical activity (11; 21) have all been recognized as contributing factors. However, the metabolic alterations involved in sarcopenia remain poorly understood and need to be clarified. Sarcopenia mainly results from a slow but continuous decrease in protein synthesis in the postabsorptive state (56), resulting from a resistance to anabolic stimuli (50) and a decrease in substrate availability.

This last process is attributed to the splanchnic sequestration of amino acids (SSAA). Splanchnic extraction of leucine (6) and phenylalanine (49) is higher in elderly subjects studied in the postprandial state than in adults. The intensity of the splanchnic sequestration of leucine has been negatively correlated with leucine plasma levels in these elderly subjects (6).

Recently, we reported the same effect in aged rats (17). Thus it appears that SSAA may be responsible for a decrease in the peripheral availability of dietary amino acids (AA), particularly for muscle protein synthesis, and is therefore a factor contributing to sarcopenia.

Since very little is known about SSAA, the aim of this study was to gain a fuller understanding of this process. SSAA is a metabolic process. It must therefore have a limit, and so can in principle be saturated. It is known that quantitative manipulation of nitrogen
supply can modulate nitrogen retention in the elderly (3). Our first working hypothesis was accordingly that SSAA could be saturated by giving a sufficient amount of protein. To test this hypothesis, we studied young and aged rats receiving either a normal-protein diet (17% protein) or a high-protein diet (27% protein) for 14 days, and we measured nitrogen balance daily.

In addition, the precise role played by the gut and liver in SSAA is unknown. Three hypotheses can be advanced to explain the age-related increase in AA utilization in the splanchnic area: (i) an increase in protein synthesis in the gut and (or) the liver, (ii) an increase in AA transamination, and (iii) an increase in AA disposal mainly via urea production. In the fed state, the liver is the main organ of AA catabolism and thus plays a major role in the control of peripheral AA availability (26). We therefore focused our attention on the third hypothesis. Thus our second working hypothesis was that the liver, via a deregulated increase in AA catabolism reflected by an increase in ureagenesis, is the main organ involved in SSAA. To test whether the liver per se is responsible for alterations in nitrogen metabolism in aged rats, we used the ex vivo isolated perfused liver (IPL) model in adult and aged rats. This ex vivo model allows simulation of in vivo conditions and standardization of experimental conditions. This model was particularly useful for our present purpose as it enabled us to study age-related alterations in hepatic nitrogen metabolism independently of potential age-related alterations in gut metabolism.
METHODS

Animals

Young adult (3-month-old) and old (24-month-old) male Sprague-Dawley rats (Charles River, Saint-Germain-sur-l’Arbresle, France) were housed in individual metabolic cages. They were kept in a controlled environment (at a constant temperature of 21°C under a 12:12 h light-dark cycle) with ad libitum access to water. Sprague-Dawley rats are commonly used in aging studies (25;46;20;36).

Experimental design (Figure 1)

During a two-week acclimatization period all the rats were given ad libitum access to a standard chow diet, A04, recommended by the breeder (Charles River) as a maintenance diet for adult and aged healthy rats and supplied by UAR (Villemoisson-sur-Orge, France). This standard chow was taken as the normal-protein diet. It contained 17% fish and vegetable protein, 3% lipids, 59% carbohydrates and 21% water, fiber, vitamins and minerals. Daily spontaneous intake was recorded.

During a two-week experimental period (Day 1 to Day 14), the rats were fed either the normal-protein diet (17% protein) or a high-protein diet (27% protein). The high-protein diet was made up using A04 chow enriched with casein (UAR) up to 27% protein. This high-protein diet contained 2.6% lipids, 51.9% carbohydrates and 18.5% water, fiber, vitamins and minerals. We set the high-protein diet at 27% protein because this level had been shown to increase protein accretion even in malnourished old rats (52) and seemed reasonable taking into account that kidney function declines with aging.

The rats were divided into four groups based on age and dietary nitrogen content during the experimental period:

- AN: young adult rats fed a normal-protein diet ($n = 7$)
AH: young adult rats fed a high-protein diet \((n = 7)\)

ON: old rats fed a normal-protein diet \((n = 6)\)

OH: old rats fed a high-protein diet \((n = 6)\)

To ensure complete food consumption, food was limited to 90% of the rats' spontaneous food intake measured during the acclimatization period \((28)\). The rats were weighed and urine was collected daily. On Day 14 of the experiment, the animals were fasted for 6 h (but with free access to water) before liver isolation and perfusion (see below). This 6 h fasting period ensured that all the rats were at the post-absorptive state and facilitated the surgical procedure. This research protocol complied with our institution's guidelines for animal care. Two of the authors, L. Cynober and C. Moinard, are authorized by the French Ministry of Agriculture to use this experimental model (authorization Nos. 005226 and 75522, respectively).

**Liver perfusion**

Rats were anesthetized under controlled isoflurane (Baxter, Maurepas, France) inhalation (Minerve, Esternay, France). Although both positive \((16)\) and adverse effects \((45)\) have been described, there is no evidence that isoflurane affects protein metabolism in rats, and it remains the best anesthetic option in this species. The livers were prepared according to Miller's technique, as previously described \((33)\). Briefly, after cannulation of the bile duct, 1 ml of saline containing 500 IU of heparin (Héparine Choay®, 25000 UI/5 mL, Sanofi, Gentilly, France) was injected into the inferior vena cava, and the portal vein was cannulated. The liver was immediately rinsed with warmed oxygenated perfusion solution and excised. The isolated liver was perfused at constant pressure through the portal vein using a recirculating system in a thermostatically controlled cabinet \((37 \pm 0.5 \degree C)\) where the perfusate was allowed to flow freely back into the tank via the hepatic veins. Portal pressure was maintained at the physiological value of 13 cm H\(_2\)O by overflow of the perfusate into the tank.
The perfusate was a Krebs-Henseleit buffer supplemented with bovine serum albumin (30 g/l; VWR, Fontenay-sous-Bois, France), glucose (8.5 mM) and calcium (2 mM) (Sigma, La Verpillière, France) and oxygenated with an O₂/CO₂ mixture (95/5 v/v). A solution of AA with antiproteolytic properties (alanine, glutamine, histidine, leucine, methionine, phenylalanine, proline and tryptophan) was also added to the perfusate to control hepatic proteolysis (9). To ensure reliable liver AA exchange in near steady-state conditions, hepatic AA extraction was compensated for from time 30 min \((t_{30})\) by a bolus plus continuous AA infusion (0.2 ml/min), which allowed approximately twice the physiological AA levels to be maintained (10). At \(t_{30}\), 0.125 mg of indocyanine green (ICG) (2.5 mg/ml, SERB, Paris, France) was also added to the perfusate for measurement of ICG extraction.

Samples of perfusate were collected at times 0, 35, 40, 45, 50, 60, 70, 80 and 90 min of perfusion for the measurement of biochemical parameters. The samples were stored at –80°C until either direct analysis or deproteinization with sulfosalicylic acid (30g/l) for AA analysis.

Bile was collected at 30-min periods, and bile flow (µl/min) was estimated gravimetrically assuming a specific mass of 1 g/ml. Portal flow (ml/min) and pH (Radiometer, Copenhagen, Denmark) of the perfusate (7.39 ± 0.05) were closely monitored throughout the experiment.

At the end of the perfusion phase, a small lobe of the liver was rapidly frozen in liquid nitrogen for measurement of intrahepatic AA. Total liver weight was then determined.

**Analytical methods**

- **Urinary parameters**

Urine samples were collected daily on a preservative (Amukin; Gifrer Barbezat, Decines, France). Urinary nitrogen output was quantified by chemiluminescence using an Antek 7000 apparatus (Alytek, Courtaboeuf, France) (13).
Urea was measured spectrophotometrically by a routine urease method on an AU600 analyzer (Olympus, Rungis, France) (5).

- **Analyses performed on perfusate samples**

  - **Biochemical parameters**

    Alanine aminotransferase (ALT) activity was measured using the AU600 analyzer according to the recommendations of the French Society for Clinical Biology (5). Urea was measured as described above.

  - **Amino acids**

    AA in the perfusate were analyzed by ion-exchange chromatography with ninhydrin detection on an AA analyzer (Jéol, Croissy-sur-Seine, France) (32).

  - **Indocyanine green**

    Indocyanine green (ICG) levels in the perfusate were measured using Nielsen's spectrophotometric method (34) with correction for blank density at 900 nm.

- **Amino acids in the liver**

  To determine free intrahepatic AA concentrations, the samples were ground and deproteinized with 10% trichloroacetic acid containing 0.5 mM EDTA (23). Supernatants were stored at –80°C until AA analysis as described above.

**Calculations**

- Nitrogen loss in urine was measured daily from D0 to D14 and nitrogen balance (NB) was calculated as the difference between nitrogen intake and urinary nitrogen output. Results are presented as daily and cumulative nitrogen balance.

- Relative nitrogen loss (RNL, in %) was calculated as the amount of nitrogen excreted (Ne) per nitrogen ingested (Ni) at D0 and D14:

  $$RNL = \frac{Ne}{Ni} \times 100$$
The ratio of urinary urea nitrogen to total nitrogen (UUN/TUN) is presented as the average value calculated from D1 to D14:

\[
UUN/TUN = \frac{((\text{urinary urea} \times 2.8))}{\text{total urinary nitrogen}} \times 100
\]

where 2.8 represents the amount of nitrogen contained in one molecule of urea.

Hepatic exchanges of substrates (F) were calculated as:

- for urea flux:

\[
F = \frac{(Lt_2 \times V_2) - (Lt_1 \times V_1)}{t_2 - t_1} / E
\]

- for AA flux:

\[
F = \left[ \frac{(Lt_2 \times V_2) - (Lt_1 \times V_1)}{t_2 - t_1} - L_i \right] / E
\]

where \( Lt_1 \) and \( Lt_2 \) are metabolite levels in the perfusion buffer at times \( t_1 \) and \( t_2 \) respectively, \( V_1 \) and \( V_2 \) are the volumes of perfusion buffer at the same times (to take into account volume variations due to sampling, evaporation and addition), \( L_i \) is the infusion rate for a given amino acid and \( E \) is the ICG extraction ratio. In a recent study (18), we demonstrated that this was a better mode of data expression than a ratio to liver weight: the ICG extraction ratio gives a better approximation of hepatic functional mass, total liver weight in aged rats being higher than in young adults irrespective of functional mass. Thus correction for this factor takes age-related changes in hepatic extraction function into account.

The data are presented as the means of the fluxes calculated over three perfusion periods (\( t_{35-45} \), \( t_{45-60} \) and \( t_{60-90} \)).

Negative values represent metabolite uptake while positive values represent metabolite release.
ICG extraction ratio:

ICG removal from the perfusate was fitted to a biexponential curve \( C(t) = Ae^{-\alpha t} + B^\beta \)
by nonlinear regression using Prism [GraphPad software, San Diego, USA]. The macroconstants \( A, \alpha, B, \beta \) were thus determined to calculate \( Cl \) (ICG clearance) and \( E \) (ICG extraction ratio), as described by Vaubourdolle et al. (48):

\[
q_0 \frac{Cl}{A/\alpha + B/\beta} \quad E = Cl/Q
\]

where \( q_0 \) is the dose of ICG and \( Q \) the perfusion flow.

Statistics

Data are presented as means ± SEM.

For nitrogen balance measured at D0 (i.e. before introduction of the high-protein diet in the AH and OH groups), a Student \( t \)-test was used to compare adult and aged rat groups.

For cumulative nitrogen balance measured from D1 to D14, a two-way (A: age and N: nutrition or their interaction: A&N) analysis of variance (ANOVA) for repeated measurements was performed to compare groups. Means were compared using the Newman-Keüls test.

For other parameters measured \textit{in vivo} and parameters measured during IPL, a two-way ANOVA was performed to discriminate between aging effects, diet effects, and their interactions (A: age and N: nutrition or their interaction: A&N). Means were compared using the Newman-Keüls test.

The StatView 5.0 software package was used. The level of significance was set at \( P < 0.05 \).
RESULTS

**Food intake and body weight**

Food intake assessed during the acclimatization period was similar for all four groups (AN: 24.34 ± 0.04 g, AH: 23.82 ± 0.05 g, ON: 23.87 ± 0.46 g, OH: 25.39 ± 0.36 g/day).

Whole-body weight was higher in old rats than in adults at D14 whatever the diet given to the rats during the experimental period (AN: 400 ± 3 g, AH: 399 ± 4 g, ON: 665 ± 37 g, OH: 623 ± 39 g, P < 0.05: O vs. A rats fed the same diet), but not different between rats of the same age. The adult rats gained weight throughout the experiment, whereas total body weight remained stable in the old rats whatever the diet (AN: 11.5 ± 0.7 g, AH: 11.8 ± 0.8 g, ON: -2.1 ± 0.4 g, OH: -1.2 ± 0.8 g, P < 0.05: O vs. A rats fed the same diet).

**Nitrogen balance (NB), relative nitrogen loss (RNL) and UUN/TUN**

On D0, all the rats were fed a normal-protein diet. In these conditions NB was lower in aged than in adult rats. This difference between adult and old rats fed a normal-protein diet was maintained throughout the experimental period (until D14) expressed either daily or as cumulative values (Figure 2).

From D1 the high-protein diet resulted in an increase in the NB of both adult and old rats and abolished the age-related initial difference in NB observed at D0. In adult rats, the increase in nitrogen balance was transient (from D1 to D9), whereas in old rats the benefit of the high-protein diet was sustained throughout the experimental period (Figure 2).

On D0, RNL was significantly higher in aged than in adult rats (A: 47.4 ± 2.5% vs. O: 70.2 ± 6.2%, P < 0.05). On D14 this difference was maintained in the old rats fed a normal-protein diet but was abolished in the rats fed a high-protein diet (AN: 42.9 ± 1.8%; AH: 60.4 ± 5.3%; ON: 65.3 ± 3.0%; OH: 69.0 ± 4.5%, P < 0.05: ON vs. AN and AH vs. AN).
UUN/TUN was similar for the four groups of rats (AN: 85 ± 4.2%; AH: 89 ± 3.2%; ON: 85 ± 5.6%; OH: 91 ± 5.7%).

**Liver characteristics and viability (Table I)**

As explained in the AA flux section, liver weight was higher in aged than in adult rats. Bile flow did not differ between groups and remained constant throughout the experiment. Portal flow was similar in all the groups. There was no diet effect on these variables. Liver viability as assessed by ALT activity was well preserved in all the groups and was not affected by age or intake of the high-protein diet.

**Hepatic metabolism**

**Urea production**

Urea production was significantly lower in aged than in adult rats (AN: 1.19 ± 0.18; AH: 1.47 ± 0.34; ON: 0.34 ± 0.03; OH: 0.32 ± 0.03 µmol/min/% of extracted ICG, \( P < 0.05 \) O vs. A rats fed the same diet). There was no diet effect on urea flux.

**AA exchanges (Table II)**

Uptake of proline, serine and arginine as well as release of glutamate, citrulline and ornithine were significantly lower \( (P < 0.05) \) in livers from aged rats than in those from adult rats. Significant variations in alanine exchanges were observed (Figure 3A), with alanine uptake in adult rats but alanine release in aged rats. There was no diet effect on AA fluxes.

**Intrahepatic AA content**

Serine and ornithine levels were significantly lower \( (P < 0.05) \), whereas branched-chain amino acid levels were significantly higher \( (P < 0.05) \) in AH group rats than in AN group rats. Ornithine and leucine levels were significantly lower \( (P < 0.05) \) in ON rats than in AN rats, but were not different in the rats given a high-protein diet (Table III).

Hepatic alanine content was 4 times higher \( (P < 0.05) \) in aged than in adult rats (Figure 3B). There was no diet effect on this hepatic accumulation of alanine.
DISCUSSION

The SSAA observed in elderly people (6;49) and in aged rats (17) may play a role in blunting postprandial stimulation of protein synthesis and therefore contribute to sarcopenia via a decrease in the peripheral availability of dietary AA. The present study was undertaken first to evaluate the response of SSAA to a high-protein diet by assessing nitrogen retention, and second to clarify the role played by the liver in SSAA by describing age-related changes in hepatic nitrogen metabolism.

In the present study the control group was formed of young adult rats (3 months old). The choice of the age of animals for an adult reference group is still a subject of debate because (i) the extrapolation of age of rats to humans is problematic, as elegantly discussed by Quinn (40), and (ii) rats grow continuously, unlike humans. Nevertheless, most authors use 3-month-old rats (1;42;8;27) or 6-month-old rats (38;43;44) as adult control groups without any major difference in protein metabolism between animals at these two ages (35).

In agreement with a previous study in rats (52), we observed that when the rats were fed a normal-protein diet, aging was associated with a decreased nitrogen balance corresponding to a lower nitrogen retention. This result does not reflect a net catabolic state in aged rats, since their weight remained stable throughout the experiment. The excreted nitrogen remained mainly as urea (85% to 90%), whatever the age of the animal, ruling out effects of acidosis possibly linked to an age-related or high-protein intake-related decrease in renal function. Although NB was lower in old rats, their percentage of nitrogen excreted per nitrogen ingested was higher than in adults. Thus AA oxidation was evidently increased in aged rats. Taken together, these data obtained in the in vivo part of our experiment thus support the concept of SSAA.
In the present study, nitrogen balance was calculated measuring only urine nitrogen output. It is well known that nitrogen absorption is not a limiting factor over a wide range of protein intake (24) except when gut function is severely compromised (e.g. short bowel syndrome). Other losses (e.g. hair) are marginal.

In adult rats, the administration of a high-protein diet led to an increase in nitrogen balance. However, this increase remained transient (from D1 to D9), and was followed by a normalization of nitrogen balance corresponding to a metabolic adaptation leading to an increase in nitrogen loss. In humans, studies (39;41) have shown that an increase in protein intake leads to increased nitrogen losses via higher amino acid oxidation, especially in the fed state. However, at D14, although the percentage of nitrogen excreted per nitrogen ingested was higher in adult rats fed a high-protein diet compared with a normal-protein diet, it remained unchanged in old rats. These data suggest that nitrogen elimination capacity is already at its maximum in the aged animals fed a normal amount of proteins, and that unlike adult rats, they do not adapt their nitrogen losses accordingly to nitrogen intake.

Thus the extra nitrogen supplied by the high-protein diet was retained by the old rats, and SSAA clearly appears to be a saturable process. This conclusion is consistent with previous observations in elderly humans (3;49) and in aged rats (4). When an amount of AA higher than that normally provided by a meal was supplied orally to adult and elderly subjects, Volpi and al. (49) observed that muscle protein anabolism and arterial phenylalanine concentration were similar in the two groups, despite higher first-pass splanchnic extraction in elderly subjects. Katsanos et al. (19) demonstrated that aging was associated with diminished protein accretion after the ingestion of a small bolus of essential AA. Thus in the study of Volpi et al. (49), the splanchnic sequestration of AA in elderly subjects may have been saturated. This would explain why despite a higher first-pass extraction of Phe, its arterial concentration was similar in elderly and young adults, and muscle protein synthesis was not affected. Also, in
elderly women, the ingestion of 80% of daily protein intake as a single meal (the so-called pulse diet), which delivers a heavy protein load, improved nitrogen balance more efficiently than when the same amount of protein was spread across four isoproteic meals (spread diet) (3). The same protocol applied to aged rats showed that the pulse diet led to higher plasma AA levels together with a restored muscle protein synthesis response to feeding (4). Taken together, these results support the hypothesis that SSAA can be saturated above a certain threshold of protein or AA supply.

The mechanism underlying SSAA, and the exact contribution of the liver and the gut in this process, remain unknown. Since the liver is the main site of AA oxidation and urea production, we examined the hypothesis that age-related SSAA is caused by hepatic changes of nitrogen metabolism using an ex vivo study model, the isolated perfused liver (IPL). One limit of our study is that we did not address the fate of AA in the intestine. This deserves further study using multi-catheter techniques (14).

Urea synthesis is subject to three types of regulation: the flux of amino acids upstream of the cycle (9), regulation by allosteric enzymes, and hormonal regulation (26). We note that our model does not allow for differences in hormonal pattern (e.g. cortisol, pro-inflammatory cytokine) between adult and old rats. During IPL, the amount of nitrogen supplied to the liver was strictly controlled and kept equivalent across all four groups. Metabolic state was also the same across groups (i.e. all the rats were in fasted state when the liver was isolated). These standardized experimental conditions enabled us to discriminate specific age-related metabolic alterations of the liver.

Metabolic fluxes (i.e. AA and urea fluxes), assessed during IPL experiment are usually expressed per gramme of wet liver. However, to allow the comparison of groups with different ages, correction by liver weight may not be sufficient, as this may not reflect hepatic functional mass in old rats. Data from previous work (18) prompted us to re-evaluate the
mode of expression of metabolic fluxes. ICG extraction ratio, a surrogate of hepatic functional mass (53), was higher in old than in adult rats. Accordingly, to take into account age-related variations in liver functions (2), our data were normalized to ICG extraction ratio. Remarkably, hepatic urea production in aged rats was lower than in adults. This result refutes our second working hypothesis that SSAA is associated with an increase in AA disposal via urea production. An alteration of the fluxes of glutamine, arginine or alanine (which are major precursors and/or inducers of urea) could potentially explain the altered ureagenesis in old rats. Aging had no effect on the hepatic flux of glutamine, ruling out its implication in the decrease in urea production in old rats. However, we observed an age-related decrease in hepatic arginine uptake. Thus not only was less substrate available for urea production, but as arginine is an allosteric activator of N-acetylglutamate synthase (26), ureagenesis was also less activated. The present study identified alanine flux as one of the major age-related hepatic alterations: alanine was taken up by the liver of adult rats, whereas it was released in old rats. In addition, hepatic alanine content was 4 times higher in old rats. It is known that the activity of the main transport system of alanine in the liver (22), the A system, is regulated by substrate availability (12). The reduction in alanine uptake could therefore be the direct consequence of intrahepatic alanine accumulation resulting from a feedback mechanism acting on alanine transport. In addition, an in vitro study (15) showed that high intracellular levels of alanine had an inhibitory effect on urea synthesis. There is a line of evidence to suggest that an inhibition of argininosuccinate synthetase is primarily responsible for the observed alanine-mediated inhibition of urea synthesis (15;47). It would now be of major interest to compare the activity of urea cycle enzymes in the liver of adult and old rats.

The decreased nitrogen balance observed in vivo in old rats fed a normal-protein diet was associated with a higher percentage of nitrogen excreted per nitrogen ingested. These results
imply that there is an age-related increase in nitrogen elimination probably occurring via an increase in dietary AA disposal. Our ex vivo results show that this process was not the consequence of an increase in urea production in aged rats.

Interestingly, ex vivo results did not show any effect of diet on urea production in either aged or adult rats, whereas an increase in urea production in response to the consumption of a protein-rich meal has previously been reported in humans (39;41). During IPL, the same amount of AA was supplied to isolated livers, whereas the rats had been fed either a normal-protein or a high-protein diet during the experimental period. Therefore, our ex vivo results confirm that urea production levels depend on the levels of AA available to the liver, as previously reported (29;37) and that there was no long-term adaptation of ureagenesis enzymes to a two-week high-protein diet in rats.

In conclusion, results from the in vivo part of the study show that old rats do not adapt nitrogen losses according to nitrogen intake. Therefore, a high-protein diet leads to improved nitrogen retention in these rats, which implies that SSAA is a saturable process responding to an increase in protein supply. Our ex vivo data demonstrate that the increase in dietary AA disposal characteristic of SSAA is not due to an age-related alteration of urea production. The liver per se does not appear to be the main organ involved in SSAA. Extra-hepatic factors may be responsible for the increase in nitrogen elimination observed in vivo in aged rats. The intestine may hold the key, since this is where arginine and glutamine, the main inducers and substrates of ureagenesis (55), are metabolized into citrulline, which does not undergo hepatic uptake (7). Intestinal citrulline production from arginine and glutamine has been shown to be dependent on protein intake (51). Taken together, these data suggest that inter-organ AA exchanges between the gut and the liver may be key factors in achieving a normal stimulation of ureagenesis during aging.
GRANTS

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### Table I: Influence of age and diet on the characteristics and viability of the isolated liver

<table>
<thead>
<tr>
<th></th>
<th>Adult Normal-protein diet</th>
<th>Adult High-protein diet</th>
<th>Aged Normal-protein diet</th>
<th>Aged High-protein diet</th>
<th>t</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>11.5±0.4</td>
<td>12.1±0.3</td>
<td>18.1±1.0*</td>
<td>18.7±1.3*</td>
<td>A</td>
</tr>
<tr>
<td>Bile flow (μl/min)</td>
<td>4.2 ±0.6</td>
<td>2.7 ±0.3</td>
<td>3.4 ±0.5</td>
<td>4.1 ±0.3</td>
<td>ns</td>
</tr>
<tr>
<td>Portal flow (ml/min/g of wet liver)</td>
<td>3.9 ±0.2</td>
<td>3.8 ±0.2</td>
<td>3.7 ±0.1*</td>
<td>3.5 ±0.1*</td>
<td>ns</td>
</tr>
<tr>
<td>ICG extraction ratio (%)</td>
<td>3.6 ±0.6</td>
<td>4.3 ±0.5</td>
<td>8.1 ±0.5*</td>
<td>11.2±1.5*</td>
<td>A</td>
</tr>
<tr>
<td>ALT (UI/l)</td>
<td>19.5±1.8</td>
<td>23.5±5.1</td>
<td>18.8±4.5</td>
<td>17.8±5.6</td>
<td>ns</td>
</tr>
</tbody>
</table>

Livers from adult or aged rats submitted to a 2-week controlled normal-protein or high-protein diet were isolated and perfused. Data are means ± SEM and represent wet liver weight at the end of perfusion and mean bile and portal flows throughout the perfusion experiment. ALT is the activity of alanine amino transferase in the perfusate at time 90 min.

A two-way analysis of variance (ANOVA) with two factors (A: age and N: nutrition or their interaction: A&N) was performed to compare groups. Means were compared using the Newman-Keüls test.

*P < 0.05 vs. adult rats fed a similar diet.

ns: not significant
Table II: Flux of selected amino acids (nmol/min/% of ICG extracted)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Adult Normal-protein diet</th>
<th>Adult High-protein diet</th>
<th>Old Normal-protein diet</th>
<th>Old High-protein diet</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>-66.9 ± 24.6</td>
<td>-66.4 ± 20.0</td>
<td>-22.5 ± 11.3</td>
<td>-27.9 ± 4.7</td>
<td>A</td>
</tr>
<tr>
<td>Glutamate</td>
<td>239.9 ± 87.0</td>
<td>233.2 ± 54.9</td>
<td>134.9 ± 27.5</td>
<td>73.5 ± 11.7</td>
<td>A</td>
</tr>
<tr>
<td>Glutamine</td>
<td>-151.6 ± 92.3</td>
<td>-99.9 ± 84.9</td>
<td>2.6 ± 45.9</td>
<td>-58.2 ± 15.7</td>
<td>ns</td>
</tr>
<tr>
<td>Alanine</td>
<td>-363.2 ± 69.4</td>
<td>-308.1 ± 71.4</td>
<td>148.5 ± 59.8*</td>
<td>89.8 ± 32.5*</td>
<td>A</td>
</tr>
<tr>
<td>Proline</td>
<td>-136.9 ± 45.5</td>
<td>-73.4 ± 42.9</td>
<td>-17.2 ± 20.2*</td>
<td>-25.8 ± 11.7</td>
<td>A</td>
</tr>
<tr>
<td>Valine</td>
<td>25.6 ± 50.6</td>
<td>70.5 ± 30.9</td>
<td>31.3 ± 18.2</td>
<td>15.4 ± 9.1</td>
<td>ns</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>16.4 ± 46.7</td>
<td>55.4 ± 28.2</td>
<td>27.5 ± 16.2</td>
<td>10.5 ± 9.2</td>
<td>ns</td>
</tr>
<tr>
<td>Leucine</td>
<td>28.9 ± 88.0</td>
<td>94.5 ± 52.7</td>
<td>40.6 ± 30.7</td>
<td>13.5 ± 15.6</td>
<td>ns</td>
</tr>
<tr>
<td>Arginine</td>
<td>-89.0 ± 16.5</td>
<td>-60.8 ± 15.1</td>
<td>-32.8 ± 7.5*</td>
<td>-30.3 ± 5.4</td>
<td>A</td>
</tr>
<tr>
<td>Citrulline</td>
<td>41.2 ± 10.8</td>
<td>36.4 ± 6.3</td>
<td>18.1 ± 1.9*</td>
<td>14.8 ± 2.8*</td>
<td>A</td>
</tr>
<tr>
<td>Ornithine</td>
<td>33.3 ± 7.5</td>
<td>26.3 ± 7.3</td>
<td>12.3 ± 1.8*</td>
<td>9.3 ± 2.0</td>
<td>A</td>
</tr>
</tbody>
</table>

Livers from adult or aged rats submitted to a 2-week controlled normal-protein or high-protein diet were isolated and perfused. Data are means ± SEM and represent the mean flux of each amino acid throughout the perfusion experiment.

Amino acids were selected according to their relevance to the aim of the study. Other amino acids did not show any relevant significant difference.

A two-way analysis of variance (ANOVA) with two factors (A: age and N: nutrition or their interaction: A&N) was performed to compare groups. Means were compared using the Newman-Keüls test.

*P.<.0.05 vs. adult rats fed the same diet.
ns: not significant

Negative values indicate uptake. Positive values indicate release.
**Table III:** Levels of selected amino acid in the liver (µmol/g of liver)

<table>
<thead>
<tr>
<th></th>
<th>Adult Normal-protein diet</th>
<th>Adult High-protein diet</th>
<th>Old Normal-protein diet</th>
<th>Old High-protein diet</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>0.30 ± 0.03</td>
<td>0.18 ± 0.02*</td>
<td>0.27 ± 0.04</td>
<td>0.29 ± 0.05*</td>
<td>A&amp;N</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.37 ± 0.17</td>
<td>2.56 ± 0.24</td>
<td>2.78 ± 0.24</td>
<td>2.14 ± 0.29</td>
<td>ns</td>
</tr>
<tr>
<td>Glutamine</td>
<td>3.90 ± 0.41</td>
<td>3.27 ± 0.35</td>
<td>4.25 ± 0.11</td>
<td>3.88 ± 0.52</td>
<td>ns</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.22 ± 0.29</td>
<td>1.86 ± 0.21</td>
<td>7.17 ± 0.77*</td>
<td>7.88 ± 1.39*</td>
<td>A</td>
</tr>
<tr>
<td>Proline</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>0.38 ± 0.01</td>
<td>0.46 ± 0.03*</td>
<td>0.34 ± 0.00</td>
<td>0.37 ± 0.02</td>
<td>N</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.27 ± 0.01</td>
<td>0.33 ± 0.02*</td>
<td>0.25 ± 0.00</td>
<td>0.27 ± 0.02</td>
<td>N</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.67 ± 0.01</td>
<td>0.76 ± 0.05*</td>
<td>0.57 ± 0.01*</td>
<td>0.61 ± 0.02*</td>
<td>A&amp;N</td>
</tr>
<tr>
<td>Arginine</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Citrulline</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.30 ± 0.02</td>
<td>0.23 ± 0.02*</td>
<td>0.22 ± 0.03*</td>
<td>0.25 ± 0.04</td>
<td>ns</td>
</tr>
</tbody>
</table>

Livers from adult or aged rats submitted to a 2-week controlled normal-protein or high-protein diet were isolated and perfused. Data are means ± SEM and represent the content of each amino acid in the liver.

Amino acids were selected according to their relevance to the aim of the study. Other amino acids did not show any relevant significant difference.

A two-way analysis of variance (ANOVA) with two factors (A: age and N: nutrition or their interaction: A&N) was performed to compare groups. Means were compared using the Newman-Keüls test.

*P < 0.05 vs. adult rats fed the same diet. #P < 0.05 vs. normal-protein-diet-fed rats of the same age.
ns: not significant

nd: not determinable
FIGURES LEGENDS

**Figure 1:** Summary of the experimental protocol

NB: nitrogen balance, Ni: ingested nitrogen, Ne: excreted nitrogen, IPL: isolated perfused liver.

Protein content was 17% in the normal-protein diet and 27% in the high-protein diet.

**Figure 2:** Effect of age on the time course of nitrogen balance (mg nitrogen/24 h) over 14 days in rats fed a normal-protein or a high-protein diet. A: cumulative nitrogen balance. B: daily nitrogen balance

Data are means ± SEM.

For nitrogen balance measured at D0 (i.e. before introduction of the high-protein diet in the AH and OH groups), a Student *t*-test was used to compare groups of adult and aged rats. *P*.<0.05 old rats vs. adults.

Data are presented as cumulative nitrogen balance measured from D1 to D14. An analysis of variance (ANOVA) for repeated measures and with two factors (age and nutrition) was performed to compare groups. Means were compared using the Newman-Keüls test.

# P < 0.05 AH vs. AN, * P < 0.05 ON vs. AN, ¤ P < 0.05 OH vs. ON

**Figure 3:** Effect of age and a high-protein diet on hepatic metabolism of alanine assessed during perfusion of isolated liver.

Data are means ± SEM and represent the flux of alanine (Figure 3A) and the content of alanine in the liver (Figure 3B).
A two-way analysis of variance (ANOVA) with two factors (age and nutrition) was performed to compare groups. Means were compared using the Newman-Keüls test.

*P < 0.05 vs. adult rats fed the same diet. An age effect was observed on both variables.
References


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Acclimatization

Normal-protein diet

-D14  -D7  D0  D14

Experimentation

Normal or high-protein diet

Determination of food intake + collection of urine

<table>
<thead>
<tr>
<th>Individual cage</th>
<th>Metabolic cage</th>
</tr>
</thead>
</table>

\[ NB = Ni - Ne \]

Figure 1
Jourdan et al
Figure 2A
Jourdan et al
Figure 2B
Jourdan et al
Figure 3
Jourdan et al