Amino Acids (1991) 1: 17–27

Amino
Acids

Flooding-dose of various amino acids for measurement of whole-body protein synthesis in the rat

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Summary. These studies were undertaken to investigate the influence of the precursor amino acid on the measurement of whole-body protein turnover by the flooding-dose method. Whole-body protein synthesis rates were estimated in 70 g rats using an intravenous injection of L-($U^{14}C$) Threonine, L-($U^{14}C$) Lysine (200 μmoles/100g; 0.15 μCi/μmol or 1000 μmoles/100 g; 0.15 μCi/μmol), L-($U^{14}C$) Phenylalanine, L-($1^{14}C$) Leucine or L-($U^{14}C$) Histidine (200 μmoles/100 g; 0.15 μCi/μmol). Forty two rats were divided into seven groups. Each group received a large dose of one of the labelled amino acids. In each group, one animal was killed every 2 min between 5 and 15 min after the injection. Whole-body protein fractional rate was determined from the slope of the linear regression of $Sb(t)$ (protein specific radioactivity) against $Si'(t) \times t$, where $Si'(t)$ is the average specific radioactivity of free tissue amino acids between 0 and t. Whole-body protein fractional synthesis rates were 41.4, 25.6, 31.1, 31.4 and 22.8%/day with threonine, lysine, phenylalanine, leucine and histidine respectively. These data suggest that the estimation of whole-body protein synthesis rate varies according to the amino acid used because of the heterogeneity of the protein pool.

Keywords: Tracer amino acids – Protein synthesis – Whole body protein

Introduction

The most widely employed approaches to estimate whole-body protein metabolism in animals and especially in man are based on the study of the kinetics of a single labelled amino acid continuously infused [1–6]. By quantifying tracer lost as expired carbon dioxide and measuring the dilution of the label in the free amino acid pool, assuming that it is equal to that in plasma, whole-body protein synthesis can be calculated. The problems and limitations of the tracer models

used in relation with infusion method have been extensively discussed [1, 7, 8]. As a further examination of the model, it is important to determine whether use of different isotope probes will allow similar quantitative estimates of the dynamics of whole-body protein metabolism. This is important if close comparisons are to be made among various studies that involve application of different tracers. It is also of particular interest to precise how each individual amino acid kinetics does accurately reflect whole-body protein kinetics under various experimental conditions. Estimates of whole-body protein kinetics measured from the flux of various amino acids are qualitatively similar in a number of cases [5, 9, 10] but some studies are in disagreement with this general finding [7, 11–13]. Nevertheless, some uncertainty remains because the model used in the infusion method does involve assumptions that may not hold under all experimental conditions or that are difficult to validate.

The purpose of the present study was to investigate the influence of the amino acid used as tracer on the estimation of whole-body protein turnover. In order to limitate as far as possible the uncertainties on protein synthesis measurements, we used the flooding dose method which is now considered as the most reliable method. Our results suggested that whole-body protein synthesis rate varied according to the tracer amino acid in normal-fed rats.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 35–40 g were obtained from Iffa Credo (Saint Germain sur L'Arbresle, France). They were housed in individual cages in a room provided with artificial illumination from 7 a.m. to 7 p.m. and kept at an ambient temperature of 21–22°C. The rats were maintained on a 12% protein diet prepared in the laboratory. The formula for this diet has been described previously (14). The food was divided in six equal meals given every four hours by an automatic device. After a 3–4 day period of acclimatation, each rat underwent cannulation of the right external jugular vein under general anaesthesia (chloral 0.4 g/100 g). The cannula was tunneled subcutaneously to the back of the head. It was flushed daily with heparinized (500 units/ml) saline (0.9% NaCl). The animals were allowed 4–5 days for recovery. They increased their weight by approximately 4 g/day during this recovery period.

Injections, collection of blood and tissue

When rats weighed approximately 70 g, they were randomly allocated to seven groups of six rats. The animals of each group were injected with a flooding dose of one of the labelled amino acids, through the intravenous cannula. In each group, one animal was killed by decapitation every 2 min between 5 and 15 min after injection. Doses were given in 1 ml/100 g body weight of 0.9% NaCl,

providing about 200 μmol and 30 μCi per 100 g body weight of L-[1-^{14}C] leucine (45–60 mCi/mmol) or L-[U-^{14}C] histidine (300 mCi/mmol) or L-[U-^{14}C] phenylalanine (450 mCi/mmol) or 200 μmol and 60 μCi per 100 g body weight of L-[U-^{14}C] threonine (200 mCi/mmol) or L-[U-^{14}C] lysine (300 mCi/mmol) or 1000 μmol and 150 μCi per 100 g body weight of L-[U-^{14}C] threonine (200 mCi/mmol) or L-[U-^{14}C] lysine (300 mCi/mmol).

Blood samples, collected in heparinized tubes, were centrifuged. Plasma samples were collected and frozen in liquid nitrogen. The digestive tract was removed rapidly and emptied with ice-cold 0.9% NaCl. The entire carcass and digestive tract were frozen promptly in liquid nitrogen. The frozen samples were stored at $-20°$C until analysed.

Measurement of whole-body protein synthesis

Plasma free amino acids were separated by ultrafiltration with an Amicon system (Amicon, Paris, France) and filtrates were diluted with 1 M pH 2.2 lithium citrate buffer, in order to get a final concentration of 0.2 M and acidified to pH 2.2. Frozen carcasses were cut into small pieces which were subsequently pulverized in a Waring Blendor. All subsequent steps for the determination of the specific radioactivity of free amino acids in plasma and acid supernatant of whole-body homogenates and the specific radioactivity of protein-bound amino acids were those previously described [15].

Fractional protein synthesis rate, FSR (defined as the percentage of tissue protein renewed each day by synthesis, i.e. %/day) were calculated as described previously [15], from the slope of the linear regression of $Sb(t)$ against $Si'(t).t$, where $Si'(t)$ is the average specific radioactivity of free amino acid between 0 and t, t the period of time between injection of the isotope and killing the animal and $Sb(t)$ the specific radioactivity of protein-bound amino acids. Individual values of $Si'(t)$ were calculated by multiplying each free amino acid specific radioactivity by the value of $Si'(t)/Si(t)$ obtained from the linear regression of $Si = f(t)$. Linear regression analysis was performed according to Snedecor and Cochran [16].

Results and discussion

Effect of the injection of large amount of various amino acids on their concentration in plasma and tissue

There was a rapid increase in plasma free amino acid levels after the injection of 200 μmoles amino acids per 100g body weight; within 5 min, the level attained a value of 2.2, 2.9, 2.3, 3.4 and 3.5 μmol/g plasma for leucine, histidine, phenylalanine, lysine and threonine respectively. These values were at least 30-times higher than the initial concentration of leucine, histidine and phenylalanine (0.07, 0.03 and 0.06 μmol/g plasma respectively). For lysine and threonine, the initial concentration being higher (0.45 and 0.27 μmol/g plasma respectively), the

increase was less important, about 10-times. Nevertheless, when the amount of lysine and threonine was 1000 μmoles per 100g body weight, their plasma concentration attained a value of 23 and 18 μmol/g plasma respectively, which was more than 50-times the basal level. Similar results were obtained with increasing flooding doses of valine in lambs [15]. Whatever the amino acid and the dose, the plasma free amino acid concentration fell rapidly after the injection. The decay curve can be approximated by an exponential function between 5 and 15 minutes after the injection (Fig. 1).

In the whole-body, levels similar to those measured in plasma were generally found. Nevertheless, with the highest dose of lysine and threonine, the tissue concentration of these amino acids was about 40% lower than the plasma level 5 minutes after the injection; but within 15 minutes, they became quite similar. There is no information on the mechanism involved in the transport of amino acids at such supraphysiological concentrations. It has been suggested that exchange diffusion is implicated instead of active transport [17]. In any case, our results show that amino acids rapidly reached equilibrium between blood and the intratissular pool in the conditions studied. Moreover it seems that, in the range of the doses used, there is no saturation of the processus, since a close correlation between the increase of the free tissue amino acid concentrations and the amount of unlabelled amino acids injected can be underlined (Fig. 2).

Phenylalanine is the most widely used amino acid as precursor in the flooding dose experiments [18–21]. But, as it has been reported [18], this amino acid is rapidly transformed in tyrosine. In our conditions, the concentration of tyrosine increased from 0.28 to 0.63 μmol/g plasma and from 0.21 to 0.58 μmol/g in the tissue between 5 and 15 minutes after the injection of phenylalanine.

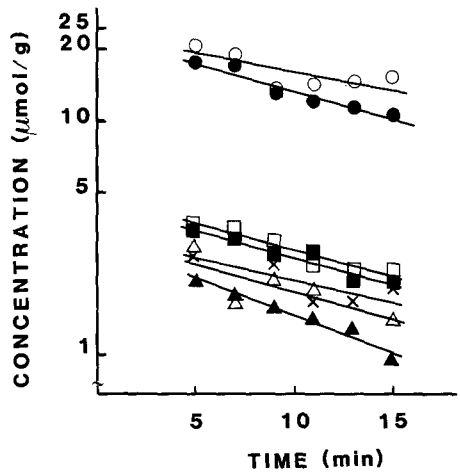

Fig. 1. Time course for plasma free amino acid concentration. Male rats, about 70 g, were injected with 200 μmoles/100 g of leucine (▲) or histidine (△) or phenylalanine (×) or lysine (□) or threonine (■) or 1000 μmoles/100 g of lysine (○) or threonine (●). Rats were killed every 2 min between 5 and 15 minutes after the injection. Plasma free amino acid contents were measured as described in Materials and methods. Data are presented as individual values

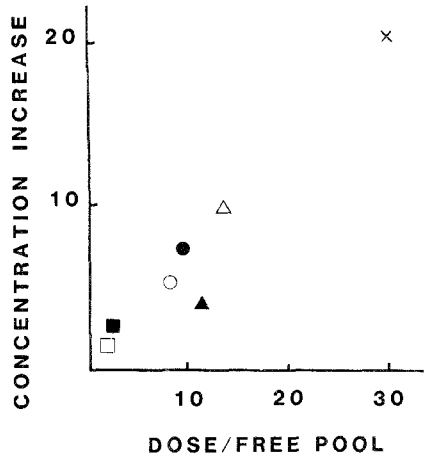

Fig. 2. Relationship between the increase in whole-body free amino acid content and the dose of unlabelled amino acids. For details of the doses, see Fig. 1. The increase in free amino acid content was calculated by dividing whole-body free amino acid content, found 15 min after the injection of unlabelled amino acids, by the initial concentration. The size of whole-body free amino acid pool was estimated by multiplying their initial concentration by tissue weight. Data are presented as individual values for leucine (▲) or histidine (△) or phenylalanine (×) or lysine (□) or threonine (■) (200 μmoles/100 g) or lysine (○) or threonine (●) (1000 μmoles/100 g)

Within 15 minutes, the level of tyrosine attained a value which was 13-times and 5-times the initial concentration in the plasma and the tissue respectively. At that time the amount of tyrosine produced in the whole-body can be estimated to be 26 μmol, i.e. about 20% of the amount of phenylalanine injected.

Specific radioactivity of free and protein-bound amino acids

The elevation of free amino acid levels produced by large injection of these compounds were sufficient to maintain a relatively constant specific radioactivity of intratissular and plasma amino acids, since the rates of decline of Sp and Si were quite small, generally less than 1%/min (Fig. 3). They were generally lower than those described in previous studies, since the amount of unlabelled amino acids used in our study were larger than those usually employed [18, 19, 22–24]. Nevertheless, a decrease of 1.2%/min in plasma and 1.7%/min in tissue for leucine and 1.6%/min in plasma for lysine and threonine with the lowest dose were observed. After injection of 200 μmol/100g of lysine and threonine, the plasma specific radioactivities did not exceed a value about 80% that of the injected amino acids and the ratios in the tissue to plasma of free lysine or threonine specific radioactivities ranged from about 70 to 87%. In the other conditions the doses of amino acids were sufficient to saturate the free pools, since free amino acid specific radioactivities in plasma and tissue were close to that of the injected amino acids (Fig. 3). It can be concluded that, whatever the amino acid, when

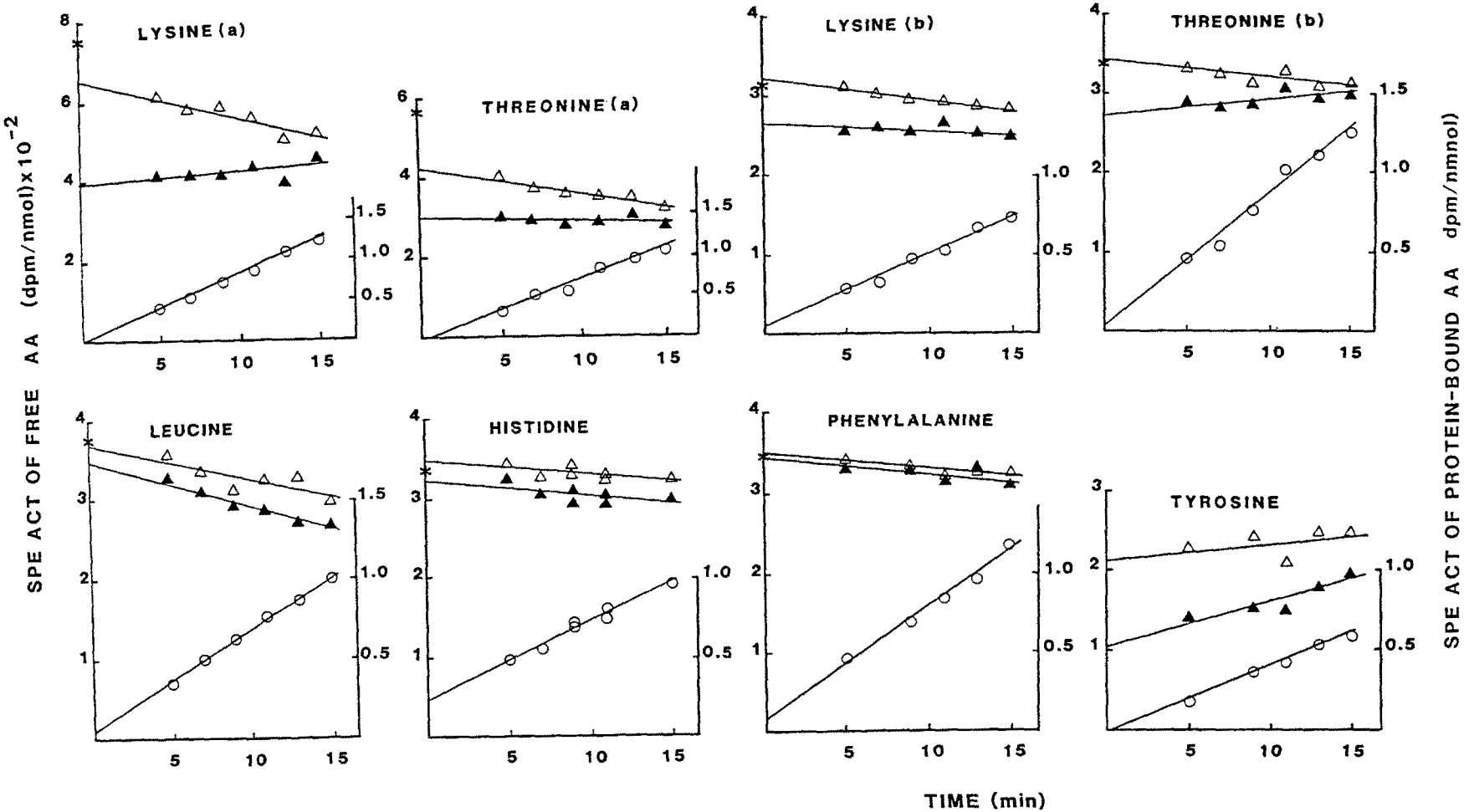

Fig. 3. Time-related changes in the specific radioactivity of different amino acids in the plasma and the whole-body of 70 g rats after injection of each [^{14}C] amino acid combined with the same unlabelled amino acid. Rats were injected with 200 μmoles/100 g of ^{14}C amino acids. For lysine and threonine, two doses were used, *a*: 200 μmoles/100 g; *b*: 1000 μmoles/100 g. The specific radioactivities of free and protein-bound amino acids in the whole-body were measured as described in Materials and methods. Data are presented as individual values. Plasma (△) and tissue (▲) free amino acid specific radioactivity and protein-bound (○) amino acid specific radioactivity. ∗: specific radioactivity of the injected solution. Lines are calculated regression lines. The equation for protein-bound amino acid specific radioactivity are $Sb = -9.9 + 74.0t$ ($r = 0.987$, $P < 0.001$) for threonine (*a*: 200 μmoles/100 g), $Sb = 36.6 + 83.5t$ ($r = 0.989$, $P < 0.001$) for threonine (*b*: 1000 μmoles/100 g), $Sb = -1.9 + 83.7t$ ($r = 0.995$, $P < 0.001$) for lysine (*a*: 200 μmoles/100 g), $Sb = 56.2 + 45.4t$ ($r = 0.988$, $P < 0.001$) for lysine (*b*: 1000 μmoles/100 g), $Sb = 93.5 + 71.1t$ ($r = 0.996$, $P < 0.001$) for phenylalanine, $Sb = -19.0 + 41.5t$ ($r = 0.996$, $P < 0.001$) for tyrosine $Sb = 47.0 + 65.0t$ ($r = 0.998$, $P < 0.001$) for leucine and $Sb = 246 + 50.1t$ ($r = 0.992$, $P < 0.001$) for histidine. The intercept with the *Y*-axis for $t = 0$ is never significantly different from zero ($P > 0.05$) except for histidine

the amount of unlabelled amino acid injected was 10-times at least the whole-body free amino acid content, the dilution due to endogenous amino acids and to the release of protein-bound amino acids in the free pool during the period of label incorporation became insignificant. Such relationships have been underlined for other amino acids such as valine [15, 25, 26].

In these conditions, the incorporation of radioactive amino acids into protein was linear within 15 minutes,even with the lowest dose of lysine and threonine and for tyrosine produced from phenylalanine. Thus for tyrosine, it can be assumed that some degree of pool flooding has been reached.

Flooding-dose method for the measurement of whole-body protein synthesis rates

To take into account the rate of decrease in free specific radioactivity, the rate of protein synthesis was calculated from the integral of $Si(t)$ between 0 and t. The measurement of both Sb and Si at various times after the injection of the flooding-dose showed that Sb increased linearly with the integral of $Si(t)$ calculated at each time point. The correlation coefficient between Sb and $Si'(t).t$ (Fig. 4) was always greater than 0.98 ($P < 0.001$). The intercept with the Y-axis for $t = 0$ was never significantly different from zero ($P > 0.05$), except for histidine. This was due to the rate of incorporation of histidine into proteins which seemed to be slower between 5 and 15 min than between 0 and 5 min. The injection of massive amounts of histidine could have inhibited protein synthesis. There was however no evidence of such an effect and, in brain, Dunlop et al. [26] have found similar FSR values with flooding doses of lysine,valine and histidine. Another possibility could be a pollution of the protein precipitate by free histidine having a high specific radioactivity. As free histidine high specific radioactivity was nearly constant, it can be assumed that the amount of radioactivity retained in protein was also constant. In this case,the use of the rate of increase of Sb to determine protein FSR allowed a good estimate. Our method of calculation provided results close to those described for leucine [27] and phenylalanine [19]. Whatever the amino acid, FSR determined either from the tissue specific radioactivity or from the plasma specific radioactivity were non significantly different ($P > 0.05$) (Table 1), except for threonine with the lowest dose, indicating that sufficient flooding has been realized.

As has been discussed in numerous reports [1, 2, 8], the uncertainties in the measurement of the specific radioactivity of the precursor represent the major obstacle in estimation of protein turnover by infusion method. Failure to achieve the same specific radioactivity intracellularly as in blood was observed in a number of tissues [1, 3, 28–30]. The large dose method is the most reliable method for estimating protein turnover. As it has been shown on perfused organs [31–33], it can be assumed that, in vivo, the specific radioactivity of amino acyl-tRNA has been maintained to values similar to those of free plasma and tissue amino acids. However, the reliability of the results required that the large amount of unlabelled amino acid does not in itself affect the result of protein

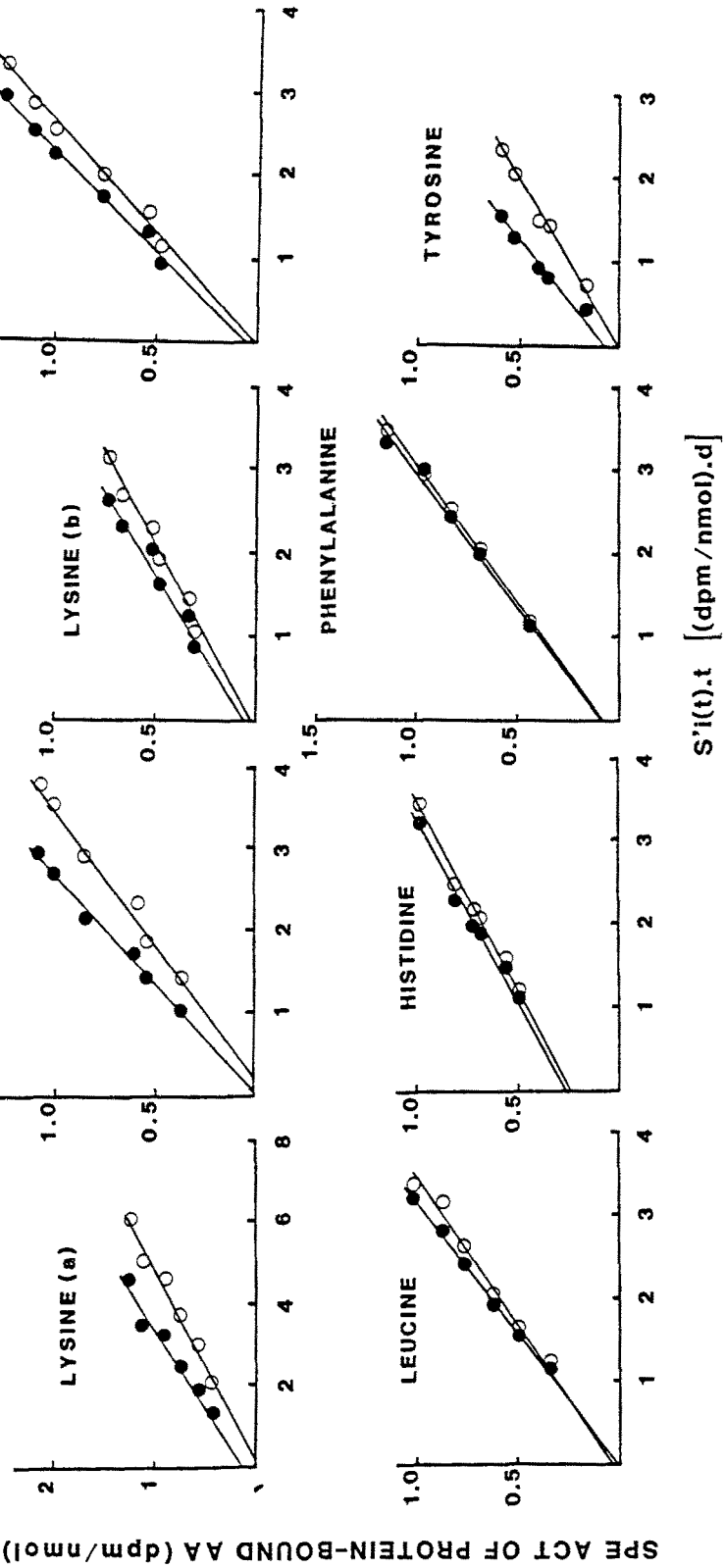

Fig. 4. Linear relationship of protein-bound specific radioactivity and $Si'(t).t$ $Si'(t).t$ is the integral of free specific radioactivity either in plasma (○) or in tissue (●) between 0 and t (see Fig. 3.) multiplied by time t for different amino acids (for calculations see Materials and methods)

Table 1. Whole-body fractional protein synthesis rate (FSR) (%/d) in 70g rats determined with different amino acids as precursor

Amino Acid	Dose	Sp	Si
Threonine	200	29.7 ± 1.9^{z}	$37.0 \pm 2.0^{x,*}$
	1000	37.6 ± 2.2^{x}	41.4 ± 2.1^{x}
Lysine	200	21.4 ± 1.9^{y}	26.0 ± 3.1^{y}
	1000	22.2 ± 1.7^{y}	25.6 ± 2.6^{y}
Phenylalanine	200	30.8 ± 1.8^{z}	31.1 ± 3.2^{z}
Leucine	200	28.0 ± 2.0^{z}	31.4 ± 0.9^{z}
Histidine	200	21.5 ± 1.3^{y}	22.8 ± 1.9^{y}

The FSR was measured after injection of either 200 or 1000 μmoles of ^{14}C amino acids/100 g body weight. Values are slope $\pm$ SE of the linear regression of Sb against S'i(t).t shown in Fig. 4. (see calculations in Materials and methods). *Si* intratissular specific radioactivity; *Sp* plasma specific radioactivity; Means in each row sharing a common superscript letter (*x*, *y*, *z*) do not differ significantly ($P > 0.05$).* value calculated from Si significantly greater than the corresponding value calculated from Sp ($P < 0.05$).

synthesis rates. This has been verified by different approaches for leucine, lysine and phenylalanine [15, 22, 23, 31–33]. FSR determined with the two amounts of lysine and threonine were not significantly different (Table 1), indicating that in our conditions, protein synthesis was not modified.

Comparison of protein synthesis rates measured with various amino acids

Different estimates of whole-body protein turnover were obtained by the different amino acids (Table 1). Lysine and histidine gave values which were about 20% lower than the similar values measured with leucine and phenylalanine. Threonine provided a result significantly greater than those obtained with the other amino acids, with the FSR 24% greater than the values determined with leucine and phenylalanine. FSR values can be also calculated from tyrosine kinetics, though flooding was not sufficient. The values obtained were 25.1 and 35.2%/day from plasma and tissue specific radioactivities respectively.

As the large dose method overcomes precursor pool specific radioactivity measurement, the present studies demonstrate that the turnover rate of whole-body protein varies according to the amino acid used as tracer. Little attention has been paid to the discrepancies between the estimations of protein synthesis obtained with various amino acid tracers. Current knowledge of whole-body protein turnover does not permit accurate or quantitative prediction of the extent to which, in its turnover, one particular amino acid is representative of others [7]. Only few studies have been devoted to the comparison of estima-

tions of protein turnover during the infusion of different amino acids [4, 5, 9, 10]. But generally, whole-body protein turnover was calculated from the plasma disposal rate of each amino acid during their infusion. With a well-balanced diet, which minimizes the oxidation of essential amino acids, the plasma disposal rate reflected their fractional content in body proteins [10, 30]. This relationship made evident the fact that, when oxidation was low, amino acids leaving the plasma pool were mainly used for protein synthesis. Consequently, FSR determined in these conditions from the plasma disposal rate of different amino acids were quite similar. Nevertheless some studies realized in various conditions seem to leave some doubt as to how the kinetics of one particular amino acid reflect the dynamics of whole-body protein metabolism [7, 11–13].

However the flooding dose method showed that different amino acids produce different estimates of whole body protein turnover. These differences could be related to the differences in the distribution of individual amino acids between fast and slow turning over proteins. Since, in the whole body, numerous proteins exist with various pool sizes, composition and half-lifes, such results could be expected. Protein FSR provided by lysine was lower than that obtained with leucine or phenylalanine. This result could indicate that the main lysine-rich proteins turn over slowly (muscle proteins) [34, 35]. On the other hand, threonine-rich proteins could have short half-lives. But there is no information on the relationship between amino acid composition of individual proteins and their turnover.

In conclusion, our study shows that whole body protein synthesis rates can differ by a factor of 2 according to the amino acid tracer. Further experiments are needed to determine the impact of changes in body composition and organ turnover on the rate of incorporation into protein of various amino acids.

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