Temporal responses of protein synthesis in human skeletal muscle to feeding

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In attempting to evaluate alterations in metabolic responses to dietary nutrients that occur in pathological conditions in man, it is first necessary to understand normal metabolic responses. The present study set out to determine the temporal responses of protein synthesis in the skeletal muscle of healthy subjects to the consumption of food. Sequential measurements of protein synthesis in quadriceps muscle were made in eight subjects by injection of 0·05 g L-[1-^{13}C]leucine/kg body-weight. The rate of protein synthesis after an overnight fast (i.e. in the post-absorptive state) was 2·2% muscle protein. After 1 h of eating, protein synthesis was unaltered (2·2%/d), but after 10 h of consuming small hourly meals the rate had risen to 2·9%/d, with a variation in response among individuals. The response of muscle to 10 h of feeding was also investigated in subjects who underwent only one measurement each, either after 10 h of eating small meals or after the same time-period when no food was given. Protein synthesis rates were only slightly elevated in the group of fed individuals (2·3%/d, *n* 6) compared with the fasted group (2.1%/d, *n* 6). Taken together the two studies suggest that in healthy adults muscle protein synthesis does not respond quickly to the influx of dietary nutrients and that even after 10 h of feeding any stimulation of protein synthesis is small.

Protein synthesis: Human skeletal muscle: Response to feeding

The response of protein metabolism to dietary intake in adult man is not clear. Measurements of whole-body protein metabolism assessed from the disappearance of labelled leucine from plasma have given rise to differing conclusions. Studies by Rennie *et al.* (1982), Hoffer *et al.* (1985) and Conway *et al.* (1988) have suggested that the rate of protein synthesis was increased in the fed state compared with fasting values. However, a range of other studies with essentially the same methodology have reported no stimulation in whole-body protein synthesis with feeding, but instead, a large decrease in whole-body protein degradation (e.g. Motil *et al.* 1981*a*, *b*; Young *et al.* 1987; Melville *et al.* 1989; Bruce *et al.* 1990).

Although these findings on whole-body protein turnover are somewhat unclear, the responses of individual tissues which can be measured directly, such as muscle, should be more conclusive, as the measurement of protein synthesis can be made directly from incorporation of a labelled amino acid. An investigation carried out by Rennie *et al.* (1982) with leucine labelled with ^{13}C has demonstrated substantial increases in muscle protein synthesis in healthy individuals who were fed compared with individuals in the post-absorptive state. Although some questions of methodology have arisen over this study, an increase of 60% in muscle protein synthesis has been confirmed in a subsequent report

(Halliday *et al.* 1988) for a group of individuals fed for 10 h compared with a group who continued fasting.

The aim of the present study was to investigate the responses of protein metabolism to feeding with a different method for assessing protein synthesis. Rather than using a continuous infusion of a tracer amount of L-[^{13}C]leucine, a method was employed in which a large amount (about 4 g) of labelled leucine was given. The advantages of this approach have been presented in detail elsewhere (Garlick *et al.* 1989) but the principle behind this procedure is to minimize uncertainty over the labelling in the precursor amino acid for protein synthesis. One of the advantages of this flooding procedure is that measurements can be completed over 90 min. It was, therefore, possible to examine the rapidity with which muscle tissue responds to nutrient intake. A preliminary report of the present study has been presented (McNurlan *et al.* 1990).

METHODS

Subjects and protocols

The present study was conducted as two experiments. In Expt 1 three sequential measurements of muscle protein synthesis rates were made in a group of eight healthy individuals, age 27 (SD 3) years, height 1·84 (SD 0·06) m, weight 74 (SD 9) kg, body mass index (BMI; weight/height^2) 22 (SD 2). The first measurement was made after an overnight fast. When the measurement was completed the subjects began a regimen of twelve standard meals consisting of milk, bread, butter and cheese which were consumed each hour. Since the present study was designed to provide information which would aid in the interpretation of clinical studies, the level of oral intake was comparable with that normally provided to patients receiving total parenteral nutrition. The dietary intake provided 10 g protein and 1308 KJ (250 kcal)/h.

In Expt 2, separate groups of six individuals were measured in the fasted state: age 28 (SD 5) years, height 181 (SD 0·09) m, weight 73 (SD 10) kg; BMI 21 (SD 1) or in the fed state: age 29 (SD 7) years, height 1·85 (SD 0·04) m, weight 73·5 (SD 5) kg, BMI 21 (SD 1). The measurements were started 10 h after the fed group had begun their hourly meals. During this 10 h the fasted group continued their overnight fast.

Both experiments were approved by the Ethical Committee of the Karolinska Institute, Stockholm.

Measurement of muscle protein synthesis

The procedure for the measurement of muscle protein synthesis rates employing the flooding technique was the same for Expts 1 and 2 and has been described in detail previously (Garlick *et al.* 1989).

L-[1^{13}C]leucine (Tracer Technologies, Somerville, MA, USA), verified for the absence of D-isomers and heat sterilized, was injected intravenously as a 20 g/l solution in saline (9 g NaCl/l) at 0·05 g/kg body weight and 20 atoms % enrichment. Arterial blood samples in Expt 1 and venous blood samples in Expt 2 (10 ml) were taken into heparinized tubes at 0, 5, 10, 15, 30, 60 and 90 min for the determination of free leucine enrichment. The enrichments of leucine and α-ketoisocaproate (KIC; the keto acid of leucine) were measured by gas–liquid chromatography–mass spectrometry on a VG 12-250 quadrupole mass spectrometer (VG Masslab, Manchester). Leucine was measured as the tertiary butyldimethylsilyl derivative and KIC was measured as the quinoxoxalinoltertiary-butyldimethylsilyl derivative under electron impact and selective ion monitoring as described by Calder & Smith (1988).

Incorporation of labelled leucine into protein over the period of measurement was

determined from percutaneous biopsies of quadriceps muscle taken at 0 and 90 min. Biopsies were frozen in liquid N_2 and stored at $-20°$ until analysis. At the time of analysis, muscle samples were pulverized between aluminium blocks cooled with solid CO_2 and precipitated with 0·2 mol perchloric acid (PCA)/l. After centrifugation the supernatant fraction was neutralized with 10 mol/l KOH and used to determine the enrichment of free leucine in the tissue. Enrichment was measured by gas–liquid chromatography–mass spectrometry on the VG 12-250, described previously, using the *N*-heptafluorobutyryl *n*-butyl ester derivative under negative chemical ionization (Mackenzie & Tenaschuk, 1974; Ford *et al.* 1985). The protein pellet from PCA precipitation was extensively washed, solubilized in 0·3 mol NaOH/l reprecipitated with PCA and hydrolysed in concentrated HCl for 24 h. HCl was removed by evaporation and leucine was separated by ion-exchange chromatography on a 600×9 mm column. The column eluate containing leucine in 0·2 mol citrate/l was put through a small (50×9 mm) ion-exchange column and eluted with 4 mol NH_4OH/l. Following removal of the NH_4OH, samples containing 3 μmol amino acid were decarboxylated with ninhydrin in sealed tubes essentially as described by Read *et al.* (1984). The enrichment of $^{13}CO_2$ was determined on a gas isotope mass spectrometer (SIRA 12; VG Isogas, Middlewich, Chester).

All measurements of enrichment were made in comparison with appropriately labelled standards.

Plasma glucose and hormone levels

Plasma glucose was determined using a glucose dehydrogenase (*EC* 1.1.1.47) method (Banauch *et al.* 1975). Radioimmunoassay was used to determine the plasma concentrations of insulin (Midgley *et al.* 1969), glucagon (Eisentraut *et al.* 1968) and cortisol (Dash *et al.* 1975).

Calculations and statistics

The calculation of the rate of protein synthesis was similar to that described previously by McNurlan *et al.* (1979), employing the equation:

$$k_s = (P_{(t)} - P_{(0)}) \times 100/A,$$

where k_s is the fraction of the protein pool synthesized each day, $P_{(t)} - P_{(0)}$ represents the increase in enrichment of leucine in protein from the beginning ($P_{(0)}$) to the end ($P_{(t)}$) of the experiment and A is the area inscribed by the precursor enrichment and the time. Precursor enrichment was assumed to be best represented by the enrichment of plasma α-ketoisocaproate from seven sequential measurements (see p. 122).

The values are expressed as mean values with their standard errors. The statistical evaluation of the data was made by two-tailed *t* tests, for paired observations in Expt 1 and unpaired observations in Expt 2. Although there was no obvious deviation from normal distribution, the data have also been analysed with the non-parametric tests, Wilcoxon's two-tailed signed rank test (for paired evaluations) and Mann–Whitney U (for non-paired evaluations), because of the rather small number of observations (*n* 6–8) involved.

RESULTS

The results of Expt 1 (Table 1) show that after an overnight fast the rate of protein synthesis in quadriceps muscle was 2·2% of the muscle protein per d, with a range of values from 1·43%/d to 3·2%/d. The rate did not respond rapidly to the onset of feeding. The measurement made during the 90 min period beginning 1 h after the first of the hourly meals indicated rates of protein synthesis that were very similar to those in the post-absorptive state. During the third measurement of protein synthesis, started 10 h after the

Table 1. *Expt 1. Rates of protein synthesis* (k_s; *%/d) in quadriceps muscle from healthy subjects in the post-absorptive state, 1 h and 10 h after eating**

Subject no.	Post-absorptive	1 h fed	10 h fed
1	2·37	1·83	3·59
2	3·21	2·74	3·20
3	1·75	1·56	—
4	2·95	2·15	2·98
5	—	2·94	3·14
6	1·80	2·45	3·75
7	1·97	2·10	2·60
8	1·43	1·48	1·05
Mean	2·21	2·15	2·90
SEM	0·25	0·19	0·34

* For details of subjects and procedures, see pp. 118–119.

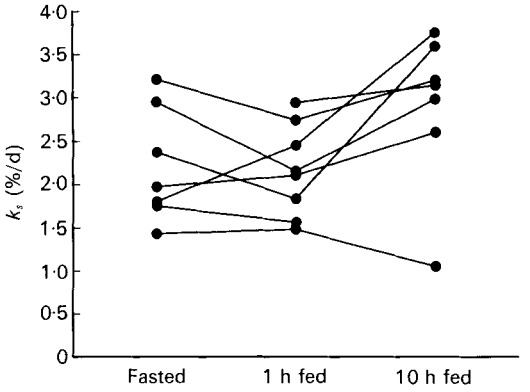

Fig. 1. Expt 1. The rate of protein synthesis in quadriceps muscle of healthy subjects measured with 0·05 g L-[1-^{13}C]leucine/kg in the post-absorptive state, after 1 h of feeding and after 10 h of feeding. For details of subjects and procedures, see pp. 118–119.

first meal, an elevation of approximately 30% was apparent. Statistical evaluation of the data with either a paired *t* test or the non-parametric, Wilcoxon's two-tailed signed rank test indicates that the values at 10 h are not significantly different from the post-absorptive values ($P > 0{\cdot}05$). The difference between the values at 1 h and those at 10 h is, however, significant ($P = 0{\cdot}05$). The data have also been examined by calculating the mean of the post-absorptive value and the value at 1 h and assessing the difference between this mean value and the value at 10 h. This difference is also not statistically significant ($P > 0{\cdot}05$). In addition, the data can be examined on an individual basis (Fig. 1), when it is apparent that four individuals did not increase muscle protein synthesis even after 10 h of feeding. One individual had a moderate increase in synthesis rate and in two individuals the rate of muscle protein synthesis was substantially increased after 10 h of eating.

By the first hour after eating began the levels of plasma glucose, insulin, glucagon and cortisol were significantly different ($P < 0{\cdot}05$) from the post-absorptive values and these changes were maintained throughout the feeding period (Fig. 2). Very transient increases were also observed in plasma insulin levels following the injection of L-[1-^{13}C]leucine. During the measurements made in both the post-absorptive state and after 1 h of eating the

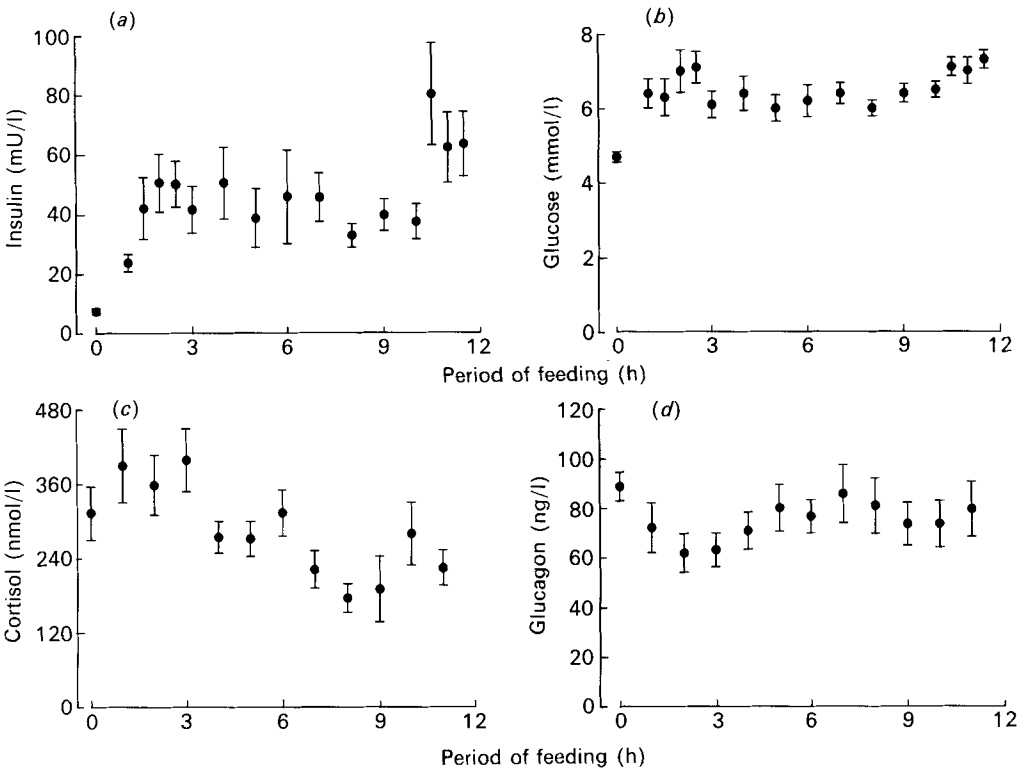

Fig. 2. Changes in plasma (*a*) insulin, (*b*) glucose, (*c*) cortisol and (*d*) glucagon of healthy subjects from the post-absorptive state to 11 h of feeding. Points are means with their standard errors represented by vertical bars. For details of subjects and procedures, see pp. 118–119.

mean increase in insulin was less than 1 mU/l. During the third measurement, made between the 10th and 11th hours of eating, plasma insulin levels were 16 mU/l higher, an increase of 33% over the value at the beginning of the hour.

The observed increase in insulin includes both the response to the injected leucine and the response to the meal which was consumed at the beginning of the hour. In studies where somatostatin was infused to alter insulin levels we observed changes in whole-body carbohydrate and fat metabolism with increases in insulin levels of 30%, but were unable to demonstrate changes in protein metabolism (McHardy *et al.* 1987; McNurlan *et al.* 1989). Moreover, in examining data from fifty measurements pooled from four other studies on muscle protein synthesis, it was not possible to demonstrate a correlation between insulin levels and rates of protein synthesis (P. Essen, unpublished results). This inability to demonstrate a stimulatory effect of insulin on protein synthesis has also been reported by others including Pozefsky *et al.* (1969) and Pacy *et al.* (1989).

Expt 1 was performed with repeat measurements in each volunteer with the intention of minimizing any data scatter resulting from differences between individuals in the rate of muscle protein synthesis. It was, however, possible that scatter in the data, resulting in an inconclusive response to feeding, had been introduced because three measurements (six biopsies) in rapid succession might have been traumatic to the volunteer or to muscle metabolism. Therefore, Expt 2 was designed to examine the response of muscle protein synthesis to feeding when subjects only underwent one measurement each. The results of this experiment, shown in Table 2, indicate an increase of only about 10% in the group of

Table 2. *Expt 2. Rates of protein synthesis* (k_s; *%/d) in quadriceps muscle from healthy subjects either fasted or fed for 10 h before measurement**

Subject no.	Fasted	Subject no.	10 h fed
1	1·72	7	2·00
2	1·25	8	1·95
3	2·38	9	1·68
4	2·22	10	2·37
5	2·43	11	2·52
6	2·60	12	3·50
Mean	2·10		2·34
SEM	0·21		0·26

* For details of subjects and procedures, see pp. 118–119.

subjects who ate for 10 h before measurement compared with the group of subjects who continued fasting for 10 h. This difference is not statistically significant ($P > 0·05$).

The rates of muscle protein synthesis for both Expts 1 and 2 have been calculated assuming that the enrichment of the leucine on tRNA, the immediate precursor for protein synthesis, can be represented by that of KIC enrichment in plasma. The rationale for this assumption is that transamination of leucine to form KIC occurs within muscle tissue, so plasma KIC reflects intramuscular leucine enrichment (Matthews *et al.* 1982), with the added advantage that the time-course of changing enrichment can be monitored without multiple biopsies. However, with the flooding procedure there is little difference in enrichment between plasma leucine and plasma KIC or free leucine from muscle, as reported previously (Garlick *et al.* 1989). In the present study, if the enrichment of plasma leucine is used in the calculation of protein synthesis, the values are only slightly different and the statistical evaluation is not altered. In Expt 1 the values are 2·12 (SD 0·63), 2·20 (SD 0·46) and 2·87 (SD 0·88) for the post-absorptive, 1 h-fed, and 10 h-fed measurements respectively, compared with 2·21, 2·15 and 2·90 calculated for the respective groups with plasma KIC enrichment. The data in Expt 2 are also similar with plasma leucine enrichment or plasma KIC enrichment used for calculation: 1·91 (SD 0·47) *v.* 2·10 for the fasted subjects and 2·20 (SD 0·6) *v.* 2·34 for the fed subjects.

Since only one muscle biopsy was taken, the enrichment of free leucine within the muscle can be compared with plasma KIC only at the end of the incorporation period. Free leucine in muscle: plasma KIC ratio was 1·04 (SD 0·04) in the post-absorptive state, 1·07 (SD 0·04) after 1 h of feeding and 1·16 (SD 0·09) after 10 h of feeding. It is unlikely that the single values reflect precisely the relationship between muscle leucine and plasma KIC throughout the incorporation period, so it would be inappropriate to calculate synthesis rates with muscle free leucine. However, it is apparent that if the actual precursor was higher than plasma KIC for the measurement made after 10 h of feeding, as suggested by the muscle leucine: plasma leucine ratio of 1·16, then the synthesis rates would be somewhat lower than the values presented and the stimulation with feeding would also be somewhat smaller than that observed with KIC enrichment.

DISCUSSION

The development of the flooding technique for measuring rates of tissue protein synthesis over short periods of time made it possible to investigate acute responses of protein metabolism. Thus, studies in young growing animals demonstrated a 43% increase in the

rate of skeletal muscle protein synthesis within 1 h of commencing feeding (Garlick *et al.* 1983). With the use of stable isotopes, the flooding technique can also be used in studies in man (Garlick *et al.* 1989) making it possible to investigate the normal, acute responses associated with the consumption of meals. We have, therefore, investigated the temporal changes in human muscle protein synthesis by sequential measurements in individuals measured after an overnight fast (i.e. post-absorptive) followed by measurements at 1 h and 10 h after they began eating.

Although demonstrable changes in plasma glucose, insulin, glucagon and cortisol occurred within the first hour (Fig. 2), there was no indication that the rate of protein synthesis in quadriceps muscle was altered (Table 1). Clearly, the rapidity of response to dietary nutrients was quite different in this group of adult subjects than that observed in young growing animals. There are two possibilities for this: either there is a species difference between rodents and man, or there is a difference in response between young, growing individuals and mature adults. Although the present study cannot differentiate between these two possibilities, studies reported by Baillie & Garlick (1991) would suggest that muscle protein synthesis in adult rats is much less responsive to acute nutritional changes than in young growing animals and that the rate of protein degradation is the more important site of regulation in the adult. Wernerman *et al.* (1985) have also reported that muscle polyribosome configuration, another index of protein synthesis, was not altered by feeding in adult human subjects. This is consistent with the observation that degradation is very sensitive to nutrient intake when measurements are made on the whole body with L-[1-^{13}C]leucine (see pp. 117–118). It seems likely, therefore, that the lack of response in muscle protein synthesis within the first 1–2·5 h of feeding was due to the adult, non-growing stature of the subjects.

With continuation of eating for 10 h, muscle protein synthesis was elevated in three of seven individuals. Although this was statistically significant by comparison with the values at 1 h of feeding ($P = 0{\cdot}05$), it was not significant compared with the fasting values. In this experiment there is considerable variability in the rates of muscle protein synthesis and it is important to examine how much of the variability might be due to imprecision of measurement and how much might be due to differences amongst individuals. The precision of measurement was assessed previously by comparing estimates of protein synthesis in quadriceps muscles from both right and left legs. The data from that study indicate that the variations between individuals cannot be accounted for by the variability of measurement (McNurlan *et al.* 1991). Alternatively, the variability among the individuals of Expt 1 can be compared with the variability reported in other studies of muscle synthesis rate involving post-absorptive subjects. In Expt 1 the coefficient of variation (standard deviation/mean × 100) was 30 %, comparable with that obtained in studies with continuous infusion (Rennie *et al.* 1982; Halliday *et al.* 1988; McNurlan *et al.* 1991) or with the flooding technique (Garlick *et al.* 1989; McNurlan *et al.* 1991; Essen *et al.* 1992). It would seem, therefore, that the variability in response among individuals arose from genuine differences rather than from imprecisions of measurement.

The observed stimulation was only 28 % for the six paired measurements, a value considerably less than the 63 % stimulation in muscle protein synthesis with feeding reported by Halliday *et al.* (1988). The study of Halliday *et al.* (1988) involved comparison of a group of volunteers who were fasted with a group of individuals that ate for 2 h before beginning an 8 h measurement of muscle protein synthesis by continuous infusion of L-[1-^{13}C]leucine. Consequently, each subject was only measured once. Each of the subjects in Expt 1, by contrast, was measured three times and it is possible that some of the subjects were not able to increase protein synthesis in response to eating because of stress responses arising from the multiple biopsies (six in total).

Expt 2 was, therefore, undertaken to examine the response to feeding in subjects who were studied only once, either after 10 h of feeding or after 10 h of fasting (in addition to the overnight fast). The longer period of fasting than that in Expt 1 was included to eliminate the possibility that the different periods of fasting associated with the 90 min flooding and 6 h continuous infusion protocols might have influenced the result. The difference between the fed group (2·34 (SD 0·21)) and the fasted group (2·10 (SD 0·26)) was not statistically significant either by Student's t test or by the non-parametric Mann–Whitney U test ($P > 0·05$). Thus, a lack of stimulation in muscle protein synthesis with feeding was observed even when the muscles had not been biopsied previously. In addition to the t tests the difference between fasting and feeding can also be assessed in terms of the confidence limits. With the data of Expt 2, and taking $P < 0·05$ as significant, the confidence interval is -24% to $+47\%$ (expressed as a percentage of the fasting value). Therefore, it is unlikely that a difference as great (63%) as that reported by Halliday *et al.* (1988) would have been undetected.

There are some differences in protocol between Expt 2 and the study of Halliday *et al.* (1988). These include the length of fasting (somewhat longer in Expt 2) and the composition (cheese sandwiches *v.* milk-based) and frequency of feeding (hourly *v.* half-hourly). However, these differences are rather small and potentially more important differences in methodology between the two studies must also be considered.

The most obvious difference is the injection of a large amount of leucine: a possible effect of leucine on protein synthesis must be considered, particularly since the values for muscle protein synthesis in the post-absorptive state measured by the continuous-infusion technique are significantly lower (1·1 %/d; Halliday *et al.* 1988; McNurlan *et al.* 1991) than rates measured by the flooding technique. Previous experiments have shown that the incorporation of a labelled amino acid given in tracer amounts can be affected by the simultaneous administration of a flooding amount of leucine. However, the incorporation of some tracers increased and the incorporation of others actually decreased (McNurlan *et al.* 1979; Smith *et al.* 1991), and it would be incorrect to conclude that this necessarily represents altered protein synthesis. Since the interpretation from experiments with tracer amounts of labelled amino acids is ambiguous, we have used other methods to investigate the possibility that the flooding amount of leucine stimulated muscle protein synthesis.

When a flooding amount of L-[1-^{13}C]phenylalanine was used to measure protein synthesis in quadriceps muscle of post-absorptive volunteers the rates obtained were very similar to those obtained with L-[1-^{13}C]leucine (McNurlan *et al.* 1991). Investigations of the effect of large amounts of phenylalanine on protein synthesis both *in vitro* and *in vivo* have not provided evidence of stimulation (Li & Jefferson, 1978; Garlick *et al.* 1980). Since it is unlikely that flooding amounts of both L-leucine and L-phenylalanine would stimulate protein synthesis to precisely the same level, the similar estimates of protein synthesis obtained with these two amino acids lends credence to the conclusion that leucine does not have a stimulatory effect on muscle protein synthesis in adult man. This conclusion is also supported by studies on limb amino acid balance which were unable to detect a stimulatory effect of leucine on phenylalanine balance or incorporation into protein (Hagenfeldt *et al.* 1980; Louard *et al.* 1990). Moreover, we have also used another method for estimating protein synthesis which does not rely on the interpretation of labelling kinetics. Changes in the aggregation of polyribosomes failed to indicate any stimulation of muscle protein synthesis by the amount of leucine used in flooding experiments (McNurlan *et al.* 1991).

If stimulation of muscle protein synthesis in adult volunteers by leucine is not likely then another possibility is a problem involving the estimate of precursor labelling with techniques involving tracer amounts of isotope. Such a condition might arise if, when post-absorptive and fed states are compared by continuous infusion of L-[1^{13}C]leucine, there

were to be a change in the source of amino acids for protein synthesis from those recycled from the degradation of protein in the post-absorptive state (relatively unlabelled) to amino acids from the plasma during feeding (relatively highly labelled). Nutrient deprivation has been shown previously to result in a change in the enrichment of tRNA relative to both intracellular and extracellular pools in cultured muscle cells (Low *et al.* 1984) and in pig muscle *in vivo* (Watt *et al.* 1989). Thus, the difference in labelling of protein between fasting and feeding might represent changes in precursor enrichment rather than changes in the rate of protein synthesis.

In conclusion, the results of the present study suggest that in adult man muscle protein synthesis was not readily stimulated by the intake of dietary nutrients despite rapid changes in plasma glucose, cortisol, glucagon and insulin. After 10 h of eating, muscle protein synthesis was elevated to a small degree (10–30%) but the difference was of marginal significance in one experiment and not significant in the second. The two experiments can, however, be considered together. The observation of a small stimulation on two independent occasions tends to support the conclusion that feeding for 10 h results in a small increase in muscle protein synthesis.

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