Myofibrillar protein turnover

Synthesis of protein-bound 3-methylhistidine, actin, myosin heavy chain and aldolase in rat skeletal muscle in the fed and starved states

Peter C. BATES, George K. GRIMBLE,* Malcolm P. SPARROW† and David J. MILLWARD
Clinical Nutrition and Metabolism Unit, Department of Human Nutrition, London School of Hygiene and Tropical Medicine, 4 St. Pancras Way, London NW1 2PE, U.K.

(Received 21 July 1982/Accepted 19 April 1983)

The turnover of 3-methylhistidine (N^{α} -methylhistidine) and in some cases actin, myosin heavy chain and aldolase in skeletal muscle was measured in a number of experiments in growing and adult rats in the fed and overnight-starved states. In growing fed rats in three separate experiments, measurements of the methylation rate of protein-bound 3-methylhistidine by either [^{14}C]- or [^3H]-methyl-labelled *S*-adenosylmethionine show that 3-methylhistidine synthesis is slower than the overall rate of protein synthesis indicated by [^{14}C]tyrosine incorporation. Values ranged from 36 to 51%. However, in one experiment with rapidly growing young fed rats, acute measurements over 1 h showed that 3-methylhistidine synthesis could be increased to the same rate as the overall rate. After overnight starvation in these rats, the steady-state synthesis rate of 3-methylhistidine was 38.8% of the overall rate. This was a similar value to that in adult non-growing rats, in which measurements of the relative labelling of 3-methylhistidine and histidine after a single injection of [^{14}C]histidine indicated that 3-methylhistidine synthesis was 37% of the overall rate in the fed or overnight-starved state. According to measurements of actin, myosin heavy-chain and aldolase synthesis in the overnight-starved state with young rats, with a variety of precursors, slow turnover of 3-methylhistidine results from the specific slow turnover of actin, since turnover rates of myosin heavy chain, mixed protein and aldolase were 2.5, 3 and 3.4 times faster respectively. However, in the fed state synthesis rates of actin were increased disproportionately to give similar rates for all proteins. These results show that (a) 3-methylhistidine turnover in muscle is less than half the overall rate in both young and adult rats, (b) slow 3-methylhistidine turnover reflects the specifically slow turnover of actin compared with myosin heavy chain and other muscle proteins, and (c) during growth the synthesis rate of actin is particularly sensitive to the nutritional state and can be increased to a similar rate to that of other proteins.

In rat skeletal muscle the overall rate of synthesis of myofibrillar proteins is slower than that of sarcoplasmic proteins (Bates & Millward, 1983), in agreement with the finding that turnover of protein-bound 3-methylhistidine (N^{α} -methylhistidine) in skeletal muscle is much slower than the overall rate of protein turnover (Millward *et al.*, 1980). Further-

* Present address: Department of Gastroenterology and Nutrition, Central Middlesex Hospital, Acton Lane, London NW10 7NS, U.K.

† Present address: Department of Physiology, University of Western Australia, Nedlands, Western Australia, Australia.

more, these studies indicate that the relative synthesis rate of the two fractions is not fixed, but varies according to the nutritional state, age and growth rate of the animals, because of disproportionate changes in the synthesis rates of the myofibrillar proteins. These findings raise the question of whether the slower turnover of the myofibril is a general feature of all individual myofibrillar proteins or a specific feature of an individual protein. In the latter case heterogeneous turnover of the myofibril would be implied. This question has been examined many times in the past in several species with different techniques, and a variety of results have been

obtained. Heterogeneous turnover rates of individual proteins have been reported by Velick (1956), Low & Goldberg (1973), Swick & Song (1974), Koizumu (1974) and Zak *et al.* (1977), whereas uniform turnover rates have been reported by Bidinost (1951), Zak *et al.* (1971), Perry (1974) and Loble & Lovie (1979). In the most recent of these reports (Loble & Lovie, 1979), data were presented from studies of synthesis rates in rabbit muscle that indicated to the authors that technical difficulties in preparing protein fractions from muscle could result in variable specific radioactivities of [^3H]tyrosine isolated from actin, and that this could account for most but not all of the previous reports showing heterogeneous myofibrillar protein turnover. However, in those studies measurements of protein synthesis were made in rabbits of various ages, so that in younger animals growth was presumably occurring and, according to our previous study (Bates & Millward, 1983), this may have masked any differences in turnover rates, since during growth there is a disproportionate stimulation of the synthesis of the slower-turning-over proteins. In the present paper we have extended our previous measurements of the turnover of protein-bound 3-methylhistidine (Millward *et al.*, 1980) to examine the extent to which muscle proteins containing this amino acid exhibit synthesis rates which differ from the overall rate, to examine specifically the turnover of the two principal myofibrillar proteins, actin and myosin heavy chain, and a non-contractile protein, aldolase, and to determine the extent to which the relative synthesis rates of these defined proteins varies according to the nutritional state of the animal. Preliminary reports of some of these findings have been presented elsewhere (Millward, 1980; Bates *et al.*, 1980, 1981; Bates & Millward, 1981).

Experimental

Animals

A total of 104 rats were used in this study, which were either Wistar:COBS or CD:COBS (Charles River, Margate, Kent, U.K.). These two albino species have similar growth rates, which are faster than that of the hooded Lister rats used by Bates & Millward (1983). Because of the necessity of measuring 3-methylhistidine, they were fed on a purified diet, free of this amino acid, in which the protein source was casein, comprising 20% (w/w) of the diet.

Experimental protocols

Expt. A: constant infusion of [methyl- ^3H]-methionine in young fed rats. L-[methyl- ^3H]-Methionine (The Radiochemical Centre, Amersham, Bucks., U.K.) was infused at 0.48 ml/h into the tail veins of two groups of male Wistar:COBS

rats weighing 102.4 ± 3.4 g (mean $\pm$ s.d.), one group for 2 h ($667.3 \mu\text{Ci/ml}$) and the second group for 5.8 h ($95.3 \mu\text{Ci/ml}$). The infusion of a greater amount of radioactivity in the group killed at 2 h enabled more precision on the measurement of incorporation at this early time. At the end of the infusion the rats were killed and the gastrocnemius and quadriceps muscles dissected from the hind limbs and frozen in liquid N_2 . Subsequently the frozen muscles were homogenized in 1.5 vol. of cold 5% (w/v) sulphosalicylic acid/10% (v/v) ethanol/0.5% thiodiglycol in water and centrifuged at $32000g$ for 20 min. The supernatant was analysed for S-adenosylmethionine specific radioactivity as described below. The precipitated protein was washed twice with 5% sulphosalicylic acid/10% ethanol/0.5% thiodiglycol, and then hydrolysed overnight in 6M-HCl with one drop of 2-mercaptoethanol, in sealed tubes. The hydrolysate was analysed for methionine and 3-methylhistidine specific radioactivities as described below.

Expt. B: constant infusion of [U - ^{14}C]tyrosine and [methyl- ^{14}C]methionine in young fed rats. Five groups of rats (male Wistar:COBS, 110.5 ± 5.6 g) were infused with L-[U - ^{14}C]tyrosine and L-[methyl- ^{14}C]methionine. A group was killed at $\frac{1}{2}$, 1, 2, 3 or 6 h. The amounts of radioisotope in the infusates were for the $\frac{1}{2}$, 1 and 2 h groups $6.25 \mu\text{Ci}$ of tyrosine/ml and $62.5 \mu\text{Ci}$ of methionine/ml, for the 3 h group $1.91 \mu\text{Ci}$ of tyrosine/ml and $19.1 \mu\text{Ci}$ of methionine/ml and for the 6 h group $1.16 \mu\text{Ci}$ of tyrosine/ml and $11.6 \mu\text{Ci}$ of methionine/ml, all groups being infused at 0.48 ml/h. At death, blood was collected and centrifuged to separate plasma, the protein from which was precipitated with an equal volume of cold 5% sulphosalicylic acid and the supernatant after centrifuging at $2800g$ for 10 min being analysed for methionine and tyrosine specific radioactivities as described below.

The gastrocnemius and quadriceps muscles were dissected out and treated as in Expt. A, except that [^{14}C]tyrosine specific radioactivity was also measured in both the supernatant and the protein hydrolysate by the method described below.

The measured specific radioactivities of the free and protein-bound amino acids in the different groups infused in these two experiments were all corrected for the different infusion rates.

Expt. C: single injection of [U - ^{14}C]phenylalanine and [methyl- ^{14}C]methionine in fed rats. In this experiment, in order to define more accurately the time course of the change in specific radioactivity of free tyrosine after a trace injection, labelled phenylalanine was injected and advantage taken of the conversion of phenylalanine *in vivo* into tyrosine, which gave a much slower change of tyrosine labelling than that observed after a single injection of a trace amount of labelled tyrosine. Twelve rats,

male CD:COBS weighing 189.6 ± 11.7 g, were divided into two groups of six. All were injected intraperitoneally with L-[U- 14 C]phenylalanine and L-[methyl- 14 C]methionine, $9.6 \mu\text{Ci}$ of each in 0.25 ml of 0.9% NaCl. One group was killed 20 min after injection and the other group 40 min after injection. The gastrocnemius and quadriceps muscles were dissected and frozen in liquid N_2 until analysis. At analysis the frozen tissue was homogenized in cold 5% sulphosalicylic acid/10% ethanol/0.5% thiodiglycol. After centrifuging, the supernatant was analysed for *S*-adenosylmethionine and tyrosine specific radioactivities as described below. The precipitate was washed twice with 5% sulphosalicylic acid, hydrolysed overnight in 6M-HCl and analysed for protein-bound tyrosine and 3-methylhistidine specific radioactivities.

Expt. D: single injection of [U- 14 C]histidine in adult female fed and starved rats. Six fed rats, female CD:COBS weighing 384.2 ± 50.8 g, which were non-growing, and six rats which were deprived of food for 24 h, weighing 361.6 ± 43.3 g, were injected intraperitoneally with $2.08 \mu\text{Ci}$ of L-[U- 14 C]histidine ($20.8 \mu\text{Ci/ml}$; 10 Ci/mol)/100 g body wt. At 60 min after injection they were killed and the gastrocnemius and quadriceps removed from one leg, combined and homogenized in 10% trichloroacetic acid. The precipitated proteins were washed until free histidine was no longer detectable and then hydrolysed in 6M-HCl overnight, and the hydrolysate was analysed for histidine and 3-methylhistidine specific radioactivities as described below.

Expt. E: measurement of synthesis of actin, myosin heavy chain, aldolase and 3-methylhistidine in fed and starved rats. The rats used in this experiment were male Wistar:COBS weighing 98.8 ± 7.8 g for the fed group and 82.4 ± 6.2 g for the 24 h-starved group. Seven fed rats were injected intraperitoneally with L-[U- 14 C]tyrosine and L-[methyl- 14 C]methionine ($4.4 \mu\text{Ci}$ and $5.8 \mu\text{Ci}$ respectively in 0.1 ml of 0.9% NaCl) and killed at 3, 6, 9, 12, 15, 20 and 40 min, one at each time point. Six other fed rats were injected with 0.2 ml of the same solution ($8.8 \mu\text{Ci}$ of tyrosine and $11.6 \mu\text{Ci}$ of methionine) and all were killed 60 min later. Thirteen rats that had been deprived of food for 24 h were similarly treated. Blood was collected from each rat and centrifuged to separate plasma, and the plasma proteins were precipitated by addition of an equal volume of 5% sulphosalicylic acid. The plasma free methionine and tyrosine were analysed on an amino acid analyser as described below to measure their specific radioactivities.

The gastrocnemius and quadriceps muscles were dissected out, combined and stored in liquid N_2 until analysed. At analysis the muscle from one leg was homogenized in cold 5% sulphosalicylic acid/10%

ethanol/0.5% thiodiglycol and the supernatant collected for measurement of *S*-adenosylmethionine, tyrosine and methionine specific radioactivities. The protein precipitate was washed twice with 5% sulphosalicylic acid, hydrolysed overnight in 6M-HCl and analysed for tyrosine and methionine specific radioactivities.

The muscle from the second leg of the rats at the 60 min time point was homogenized in a low-ionic-strength buffer (0.025 M- NaH_2PO_4 /0.025 M- Na_2HPO_4 , pH 7.0) and sarcoplasmic and myofibrillar protein fractions were prepared as described previously (Bates & Millward, 1983) except that sodium salts were used in place of potassium salts, since sodium dodecyl sulphate was to be added at a later stage. From the sarcoplasmic fraction, soluble in the low-ionic-strength buffer, aldolase was prepared as described by Scopes (1977). A fraction containing the aldolase was precipitated from the low-salt buffer solution by addition of $(\text{NH}_4)_2\text{SO}_4$ (45–60% satn. fraction). After dialysis overnight against Mops (3-morpholinepropanesulphonic acid)/KOH, pH 7.2, aldolase was purified by affinity elution from a column (1.1 cm $\times$ 3 cm) of CM-cellulose (CM-52; Whatman, Maidstone, Kent, U.K.) by addition of fructose 1,6-bisphosphate to the buffer. The aldolase was tested for purity by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and only a single band was detected. The aldolase from each muscle was precipitated out, by addition of an equal volume of 10% trichloroacetic acid, and hydrolysed in 6M-HCl. The hydrolysate was analysed for tyrosine specific radioactivity on an amino acid analyser as described below.

The myofibrillar fraction was dissolved in 8M-urea/2% sodium dodecyl sulphate, in as small a volume as possible, generally about 5 ml. The proteins were then fractionated by gel filtration. A column (2.2 cm $\times$ 90 cm) of Sepharose CL6B (Pharmacia) was equilibrated with buffer (0.5% sodium dodecyl sulphate/0.2M-NaCl/0.1M-sodium pyrophosphate, pH 7.0) and the whole of the myofibrillar fraction was run on to it. The buffer flow rate was 8 ml/h, the column effluent was monitored at 280 nm with an Isco UA4 absorbance detector, and 30 min fractions were collected. With this system sufficient resolution could not be obtained to separate discrete peaks completely, since there seemed to be slight residual binding between the proteins, whatever buffer was used (Fig. 1). However, by taking fractions from only the centres of the peaks, myosin heavy chain and actin could be separated from each other without contamination, as shown by subsequent sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The myosin heavy chain (fraction 2, Fig. 1) and the actin (fraction 4, Fig. 1) were precipitated with trichloroacetic acid, hydrolysed in 6M-HCl, and the HCl was then removed by

Fig. 1. Gel filtration of actomyosin from rat skeletal muscle

Actomyosin in 8 M-urea/2% sodium dodecyl sulphate was run on Sepharose CL6B as described in the text. The A_{280} was monitored with a full-scale deflection of 2.0. Individual fractions collected from the regions marked as 1–5 were pooled. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis showed that fraction 2 contained myosin heavy chain, free of actin, and fraction 4 contained actin, free of myosin heavy chain.

evaporation, under vacuum, to dryness. From the myosin heavy chain tyrosine, methionine and trimethyl-lysine were isolated and from the actin tyrosine, methionine and 3-methylhistidine were isolated as described below, and their specific radioactivities measured on an amino acid analyser. In addition, a sample of myofibrillar protein was hydrolysed and the specific radioactivity of 3-methylhistidine was measured.

Amino acid analyses

The amino acids in these experiments were separated by automated ion-exchange chromatography with a Locarte analyser with a 0.9 cm × 50 cm column of resin, mean bead diameter 7 μm, fitted with a split-stream pump for fraction collection. Methionine, tyrosine and phenylalanine were eluted with lithium citrate buffers containing 0.3 M-Li⁺, pH 3.55, at 50°C rising to 70°C.

For 3-methylhistidine and histidine, before the mixture was run on the analyser a partial purification was performed by the method of Haverberg *et al.* (1974). A column (0.7 cm × 8.5 cm) of AG 50 (X8) cation-exchange resin (Bio-Rad Laboratories, Watford, Herts., U.K.) was prepared. Up to 8 ml of hydrolysate containing up to 200 mg of hydrolysed protein in 6 M-HCl was loaded on this column, or, if greater recovery was required, the HCl was removed by evaporation under vacuum and the sample redissolved in 0.2 M-pyridine before loading. The

column was washed with 12–20 vol. (40–65 ml) of 0.2 M-pyridine, the larger volumes being used when the HCl was not removed from the sample; 3-methylhistidine was then eluted with 2 column vol. of 2 M-pyridine, and the column was regenerated with 5 vol. of 5 M-pyridine and re-equilibrated with 10 vol. of 0.2 M-pyridine. The sample in 2 M-pyridine was evaporated to dryness under vacuum and loaded on the amino acid analyser. 3-Methylhistidine was eluted with sodium citrate buffers (1.0 M-Na⁺, pH 4.5) at 45°C. Histidine was eluted with sodium citrate buffers (0.6 M-Na⁺, pH 4.5). Sufficient histidine was eluted from the pyridine column to enable us to measure its specific radioactivity. Trimethyl-lysine was eluted with sodium citrate (0.35 M-Na⁺, pH 5.85) at 28° rising to 60°C.

Measurement of S-adenosylmethionine

For the infusion experiments, A and B, this was measured as described by Grimble (1981) by ion-exchange chromatography. A column (0.6 cm × 4.2 cm) of 3 μm-bead-diameter cation-exchange resin (Locarte) was washed with 4 M-ammonium citrate, pH 12, then equilibrated with the running buffer (1.38 M-NH₃/10% ethanol/0.5% thiodiglycol, adjusted to pH 4.2 with citric acid, kept cold while mixing to avoid loss of NH₃). Then 1 ml of the sample in 5% sulphosalicylic acid/10% ethanol/0.5% thiodiglycol was loaded with 0.5 ml of 0.05 M-ammonium citrate before and after it. The column effluent was monitored by u.v. absorbance at 254 nm on an ACS spectrophotometer (Applied Chromatography Systems, Luton, Beds., U.K.), and fractions were collected for radioactivity counting. S-Adenosylmethionine quantities were estimated from peak areas on a chart recorder connected to the spectrophotometer by using a molar absorption coefficient of 14040 (Grimble, 1981).

For the single-injection experiments, C and E, where intracellular methionine specific radioactivity was also required, a modification of this method was developed, based on a method of Eloranta *et al.* (1976). A column (0.55 cm × 1 cm) of Whatman P11 phosphocellulose was set up, in a Pasteur pipette with a glass-fibre plug, and equilibrated with 1 mM-HCl. The sample in 5% sulphosalicylic acid was run on to this and washed through with 5 ml of 1 mM-HCl. This eluate, containing the methionine, tyrosine and phenylalanine, was evaporated to dryness and loaded on the Locarte amino acid analyser. The P11 column was washed with a further 20 ml of 50 mM-HCl and 0.4 ml of 0.7 M-NH₃, adjusted to pH 4.2 with citric acid. The S-adenosylmethionine was then eluted with 0.45 ml of the 0.7 M-ammonium citrate buffer, pH 4.2, 0.05 ml of 5% thiodiglycol in ethanol was added, and the mixture was run on a shortened column (0.6 cm × 2.2 cm) of 3 μm cation-exchange resin with 1.38 M-

ammonium citrate buffer. Several millilitres of sample could be loaded on to the P11 column, so this had the additional advantages of concentrating the *S*-adenosylmethionine and shortening the run time for analysis. Since the P11 phosphocellulose columns were so small, these were disposed of after a single use.

Results

In the first infusion experiment, A, with [*methyl*-³H]methionine, the specific radioactivity of the intracellular free methionine could not be measured. It was found that there was a contaminating radioactive peak which ran immediately before methionine on the amino acid analyser but did not react with ninhydrin. However, the specific radioactivities of muscle intracellular *S*-adenosylmethionine, protein-bound methionine and protein-bound 3-methylhistidine were measured, and these results are presented in Table 1, Expt. A.

The 2 h and 5.8 h values for the *S*-adenosylmethionine specific radioactivities were not significantly different from each other, so the mean

value was taken as the precursor labelling, $\bar{S}_i$. The 3-methylhistidine synthesis rate, K_s , could be calculated from the expression

$$K_s = \frac{\Delta S_b}{\bar{S}_i \cdot t} \quad (1)$$

where ΔS_b was the increase in product labelling (i.e. protein-bound 3-methylhistidine) during the time t between the two measurements. The value of K_s was 3.55%/day.

One histidine residue is methylated in each molecule of actin formed, and in myosin of white muscles, but not of red muscles, one histidine residue per heavy chain is also methylated (Trayer *et al.*, 1968; Huszar & Elzinga, 1971). Given the fact that the relative amounts of myosin heavy chain and actin in muscle are about 2:1, equivalent to molar ratios of 1:2.5 (see Waterlow *et al.*, 1978, chapter 15), between 70 and 100% of 3-methylhistidine occurs in actin according to the relative amounts of the different polymorphic forms of myosin heavy chain present. Thus the synthesis rate of protein-bound 3-methylhistidine reflects primarily the synthesis rate of actin.

Table 1. *Synthesis rates of protein-bound 3-methylhistidine and the rate of mixed muscle protein synthesis in young rats*

Expt. A: measurements were made after the constant intravenous infusion of L-[*methyl*-³H]methionine in fed rats (102 g) infused for 2.08 or 5.8 h. Expt. B: measurements were made after the constant intravenous infusion of L-[¹⁴C]tyrosine and L-[*methyl*-¹⁴C]methionine in groups of rats for $\frac{1}{2}$, 1, 2, 3 and 6 h. Expt. C: measurements were made after the single trace injection of L-[¹⁴C]phenylalanine and L-[*methyl*-¹⁴C]methionine. In each case 3-methylhistidine synthesis was calculated from the labelling of protein-bound 3-methylhistidine (3MeHis) and that of its precursor *S*-adenosylmethionine (AdoMet) during the specified time as described in the Experimental section. In Expt. A the overall rate of protein synthesis is calculated from the labelling of protein-bound methionine and that of *S*-adenosylmethionine, since labelling of free methionine was not measured. In Expt. B the overall rate is calculated from the labelling of both protein-bound and free tyrosine and methionine during the entire period of the infusion, or between 2 and 6 h, when a plateau labelling of the precursor had been reached and linear incorporation was occurring (see Fig. 2). In Expt. C the overall rate was calculated from the incorporation of [¹⁴C]tyrosine, formed *in vivo* from the injected [¹⁴C]phenylalanine, into protein between 20 and 40 min, assuming a linear fall in tyrosine labelling. The results are expressed as means $\pm$ S.D. for the numbers of rats described in the Experimental section.

Expt.	Time (min)	Precursor labelling (d.p.m./nmol)		Product labelling (d.p.m./nmol)		Synthesis rate (%/day)	
						3MeHis	Mixed protein
A	125	AdoMet	24 905 $\pm$ 2401	3MeHis	49.9 $\pm$ 5.6	3.55	9.89
		Methionine		Methionine	224.4 $\pm$ 23.2		
	348	AdoMet	28 000 $\pm$ 4494	3MeHis	195.3 $\pm$ 15.4		
		Methionine		Methionine	629.9 $\pm$ 49.3		
B	0–360	AdoMet		3MeHis		4.92 $\pm$ 0.65	10.48 $\pm$ 0.40
		Methionine		Methionine			
		Tyrosine		Tyrosine			
	120–360	AdoMet		3MeHis		4.51	10.50
C	20	AdoMet	245 $\pm$ 16	3MeHis	0.151 $\pm$ 0.035	6.77	13.26
		Tyrosine	24.6 $\pm$ 1.7	Tyrosine	0.0379 $\pm$ 0.0036		
	40	AdoMet	278 $\pm$ 28	3MeHis	0.397 $\pm$ 0.079		
		Tyrosine	15.5 $\pm$ 1.4	Tyrosine	0.0748 $\pm$ 0.0070		

Although the overall synthesis rate could not be calculated without the intracellular free methionine labelling, given that labelling of methionine and *S*-adenosylmethionine is similar (as shown below), an approximate synthesis rate can be calculated from the labelling of *S*-adenosylmethionine and protein-bound methionine. This was 9.89%/day, suggesting that the rate of 3-methylhistidine synthesis was considerably slower than the overall rate.

The second constant-infusion experiment, B, defined the rise to plateau of specific radioactivities of intracellular free tyrosine, methionine and *S*-adenosylmethionine and plasma free tyrosine. Plasma methionine again could not be resolved from contaminating radioactive peaks, but muscle free methionine could, by shortening the fraction collection time to 3 min. The resulting curves for free and protein-bound labelling are shown in Figs. 2(a) and 2(b). Each curve for the precursor labelling (*S*) with time (*t*) can be approximated to a single exponential equation of the form:

$$S = S_{\max} (1 - e^{-\lambda t})$$

so the rate constant for the rise to plateau (λ) can be calculated (Waterlow & Stephen, 1967). The values were 27 and 37 days⁻¹ for *S*-adenosylmethionine and methionine, though both achieved the same plateau values. Values of λ for tyrosine were 70 and 30 days⁻¹ for plasma and intracellular respectively.

From the specific radioactivities of protein-bound (*S_b*) tyrosine, methionine and 3-methylhistidine and appropriate precursors (*S_i*) shown in Fig. 2, synthesis rates (*K_s*) can be calculated either from eqn. (1) described above or from eqn. (2) (Garlick *et al.*, 1973):

$$\frac{S_b}{S_i} = \frac{\lambda_i}{\lambda_i - K_s} \cdot \frac{(1 - e^{-K_s t})}{(1 - e^{-\lambda_i t})} - \frac{K_s}{\lambda_i - K_s} \quad (2)$$

The advantage of the first method of calculation in the present experiments is that synthesis rates can be calculated between any time intervals during the infusion, and any changes in the synthesis rate during the infusion would be indicated. On the other hand, only a single value is calculated for each group of rats. In the second method a synthesis rate can be calculated for each individual animal at 6 h. Values for synthesis rates of mixed muscle protein based on tyrosine and methionine as well as synthesis rates for 3-methylhistidine are shown in Table 1, Expt. B. It should be noted that, because the synthesis rates were calculated from the specific radioactivities of the individual amino acids isolated from the free and bound pools, any isotope exchange, if it occurred at all, would not affect the results. Tyrosine and methionine give very similar results for the synthesis rates of total protein in muscle, about 10%/day. The synthesis rate of 3-methylhistidine was very much lower, at 4.9%/day.

Fig. 2. Time course of labelling of free and protein-bound amino acids during a constant intravenous infusion of [*U*-¹⁴C]tyrosine and [*methyl*-¹⁴C]methionine in Expt. B (a) Labelling of free tyrosine in plasma (O) and muscle (Δ), and protein-bound tyrosine (●). Values are means $\pm$ 1 s.d. for at least four rats. The curves drawn are single exponentials of the form $S = S_{\max} (1 - e^{-\lambda t})$ with values of the rate constants (λ) of 70 days⁻¹ for free tyrosine in the plasma and 30 days⁻¹ for free tyrosine in muscle. (b) Labelling of free methionine (O) and *S*-adenosylmethionine (Δ) and of protein-bound methionine (●) and 3-methylhistidine (■) in muscle during a constant infusion of [*methyl*-¹⁴C]methionine. Values are means $\pm$ 1 s.d. for at least four rats. The curves drawn are single exponentials for the form $S = S_{\max} (1 - e^{-\lambda t})$, where $\lambda = 37$ days⁻¹ for methionine and 27 days⁻¹ for *S*-adenosylmethionine.

Each of these first two measurements was made in the fed state in growing rats, when synthesis would be expected to be faster than degradation. However, the synthesis rates of 3-methylhistidine

were remarkably low, especially since both groups of rats were growing at about 3–4%/day before the infusions, and net 3-methylhistidine synthesis should have been occurring at this rate. One explanation of this low rate may be that during the 6 h of the infusions, while these rats were not actually feeding, synthesis rates were less than optimum.

To explore this hypothesis, measurements were made over a shorter time period in fed rats, so that there was less chance of a decrease in protein synthesis. Thus a single-injection experiment was performed in which the overall rate of synthesis and that of 3-methylhistidine were measured up to 40 min after the injection. Preliminary measurements showed that after a single injection of [*methyl*-¹⁴C]methionine, although the methionine specific radioactivity increased and decreased very rapidly, the *S*-adenosylmethionine labelling changed more slowly, and the change between 20 and 40 min was fairly small. Since the labelling of *S*-adenosylmethionine was increasing during the first 20 min after the injection, the protein-bound 3-methylhistidine should show the maximum rate of increase in labelling between 20 and 40 min. The rate of change of tyrosine labelling is very rapid after a single trace injection, so it was decided to slow this down by injecting labelled phenylalanine. Phenylalanine is converted into tyrosine in the liver (Kaufman, 1970), so, if labelled phenylalanine is injected, labelled tyrosine should be produced in the liver, and should exchange with the plasma pool and appear in the muscle intracellular free pool. This should modulate the change in tyrosine specific radioactivity such that, whereas phenylalanine labelling will show a rapid rise and fall in the muscle, tyrosine should show much more gradual changes, and this was confirmed in a preliminary experiment. The results are shown in Table 1, Expt. C. The labelling of tyrosine decreased by 37% between 20 and 40 min, and this fall was assumed to be linear with time, so that the average value for tyrosine labelling over this time was assumed to be the mean of the two measured values. Although there was a 13% increase in the labelling of *S*-adenosylmethionine, the mean value was not significantly different from either value. The calculated rates of synthesis were 13.3%/day for the overall rate and

6.7%/day for the 3-methylhistidine rate. Although both of these rates were a little higher than in the constant-infusion experiments, the ratio of synthesis rates (0.50) was not different from that in Expt. B.

The main finding from all these results is that 3-methylhistidine synthesis in muscle of young growing rats is much slower than the overall rate, as we previously reported in non-growing adult female rats (Millward *et al.*, 1980). In all of these experiments it was assumed that *S*-adenosylmethionine was not compartmented intracellularly, so that the measured specific radioactivity was similar to that pool which methylated protein-bound histidine. Although there is little evidence to suggest that *S*-adenosylmethionine is compartmented, it was decided to make additional measurements with a different method for measuring 3-methylhistidine synthesis. Labelled histidine was injected and the relative labelling of histidine and 3-methylhistidine was measured. Assuming that the incorporation of histidine is equivalent to the overall rate of protein synthesis (in the same way as methionine and tyrosine incorporation give the same overall synthesis rates) and that the histidine methylated originates from the same precursor pool as the rest of protein-bound histidine and is methylated at early times after peptide-bond synthesis (Morse *et al.*, 1975), then the ratio of labelling of 3-methylhistidine to histidine should indicate the ratio of synthesis of 3-methylhistidine to the overall rate.

In this experiment it was decided to make these measurements in a group of adult non-growing female rats so that steady-state synthesis rates could be determined. Furthermore, in order to be certain of a steady state, measurements were also made after overnight starvation. The results are shown in Table 2. Ratios of 3-methylhistidine to histidine labelling after 60 min were 0.388 and 0.355, very similar to the ratios observed in Expt. A but somewhat lower than in Expts. B and C. Given that the synthesis rates of mixed muscle proteins in adult female rats is 4.18%/day (Brown *et al.*, 1981), this indicates 3-methylhistidine synthesis to be about 1.6%/day.

Although all these studies indicate that 3-methylhistidine synthesis is slower than the overall rate of protein synthesis in muscle, they do not indicate

Table 2. Relative synthesis rates of protein-bound 3-methylhistidine and total protein in non-growing adult female rats in Expt. D

Experimental details are described in the text. The relative specific radioactivities 1 h after a single injection of labelled histidine will be equal to the relative synthesis rates (K_s). Values are means $\pm$ S.D. from six rats.

	Histidine (d.p.m./nmol)	3-Methylhistidine (d.p.m./nmol)	K_s for 3-methylhistidine K_s for total protein
Fed	0.160 $\pm$ 0.006	0.0622 $\pm$ 0.0100	0.388 $\pm$ 0.054
24 h-starved	0.151 $\pm$ 0.011	0.0537 $\pm$ 0.0105	0.355 $\pm$ 0.060

whether this slower turnover rate is a feature of all myofibrillar proteins or just of those containing 3-methylhistidine. To examine this question, relative synthesis rates of myosin heavy chain and actin as well as aldolase, a soluble protein, were measured in fed young rats and after overnight starvation by means of a single injection of labelled tyrosine and [*methyl*- ^{14}C]methionine.

The gel filtration did not give complete separation of the contractile proteins, as is apparent in Fig. 1. The A_{280} indicated a large initial peak, which was most probably RNA, since its A_{260}/A_{280} ratio was much higher than that of any other fraction and gel electrophoresis of this fraction indicated that little protein was present. Fraction 2 collected from the middle of the second peak was mainly myosin heavy chain, but, although there was no actin present in this fraction, there were several other proteins, amounting to about 20% of the total. Fraction 3 included myosin heavy chain, regulatory proteins and actin, and fraction 4 was mainly actin, with no trace of myosin heavy chain but with small quantities of low-molecular-weight proteins. Fraction 5 contained proteins of lower molecular weight than actin.

Fractions 2 and 4 were hydrolysed and the specific radioactivities of methionine, tyrosine, 3-

methylhistidine (fraction 4) and trimethyl-lysine (fraction 2) were determined. Given the lack of purity of these two fractions, synthesis rates based on methionine and tyrosine labelling will be approximate, whereas the 3-methylhistidine and trimethyl-lysine can be confidently assumed to be derived solely from actin and myosin heavy chain. Calmodulin contains trimethyl-lysine, but, since its molecular weight is only 16 500 (Klee *et al.*, 1980), it would only be present in fraction 5, the post-actin peak.

The actual synthesis rates of these two proteins and of aldolase and mixed muscle protein were calculated from the labelling of the protein-bound amino acid and the area under the appropriate precursor specific-radioactivity time course (Figs. 3a–3e).

The synthesis rates of the proteins were calculated from the equation

$$K_s = \frac{S_b}{\int_0^t S_1} \quad (3)$$

where the value of $\int_0^t S_1$ was determined graphically. The results are shown in Table 3.

The precision with which specific radioactivities can be determined by preparative ion-exchange

Table 3. *Synthesis rates of total protein, myosin, actin and aldolase in skeletal muscle of fed and 24 h-starved 100 g rats (Expt. E)*

Rates are calculated from the labelling of the various products (S_b) isolated from the muscle proteins 60 min after the injection of [^{14}C]tyrosine and [*methyl*- ^{14}C]methionine, divided by the area under the appropriate precursor time course ($\int_0^t S_1$). Since a single value for the magnitude of S_1 was used to calculate K_s for each individual value of S_b , the s.d. shown for K_s is that due to the variation of S_b alone (shown as mean $\pm$ s.d. for six measurements.) Abbreviations used: Tyr, tyrosine; Met, methionine; AdoMet, S-adenosylmethionine; Me₃Lys, trimethyl-lysine; 3MeHis, 3-methylhistidine.

Protein	Precursor		$\int_0^{60} S_1$ (d.p.m. $\cdot$ min $\cdot$ μmol^{-1})	Product	S_b (d.p.m./ μmol)	Synthesis rate	
						(%/day)	Starved/fed
Mixed	Tyr	Fed	10900	Tyr	1.06 ± 0.14	14.0 ± 1.8	0.61
		Starved	11300		0.67 ± 0.12	8.5 ± 1.5	
	Met	Fed	18700	Met	1.78 ± 0.18	13.7 ± 1.4	0.62
		Starved	23400		1.44 ± 0.19	8.9 ± 1.2	
Aldolase	Tyr	Fed	10900	Tyr	1.31 ± 0.19	17.3 ± 2.5	0.54
		Starved	11300		0.73 ± 0.20	9.3 ± 2.5	
Myosin	Tyr	Fed	10900	Tyr	1.35 ± 0.26	17.9 ± 3.4	0.57
		Starved	11300		0.80 ± 0.29	10.2 ± 3.7	
	AdoMet	Fed	15060	Me ₃ Lys	1.55 ± 0.65	14.9 ± 6.2	0.45
		Starved	10700		0.50 ± 0.19	6.7 ± 2.5	
	Met	Starved	23400	Met	1.55 ± 0.31	9.5 ± 3.9	
Actin	Tyr	Fed	10900	Tyr	1.27 ± 0.31	16.8 ± 4.0	0.33
		Starved	11300		0.45 ± 0.18	5.7 ± 0.9	
	AdoMet	Fed	15060	3MeHis	1.61 ± 0.45	15.4 ± 4.3	0.18
		Starved	10700		0.20 ± 0.12	2.71 ± 1.4	
	Met	Starved	23400	Met	0.82 ± 0.16	5.1 ± 0.8	
Actomyosin	AdoMet	Fed	15060	3MeHis	1.44 ± 0.40	13.8 ± 4.5	0.24
		Starved	10700		0.25 ± 0.10	3.3 ± 1.3

Fig. 3. Time course of labelling of tyrosine, methionine and *S*-adenosylmethionine after a single trace injection of $[U-^{14}C]$ tyrosine and $[methyl-^{14}C]$ methionine in fed and starved rats in Expt. E

Each point is for a single animal, except at 60 min where the mean $\pm$ 1 s.d. for six rats is shown. (a) Time course of labelling of tyrosine in muscle (O) and plasma (Δ) in fed rats. (b) Time course of labelling of tyrosine in muscle (O) and plasma (Δ) in rats starved for 24 h. (c) Time course of labelling of methionine in plasma (Δ) and muscle (O) in fed rats. (d) Time course of labelling of methionine in plasma (Δ) and muscle (O) in rats starved for 24 h. (e) Time course of labelling of *S*-adenosylmethionine in muscle from fed (O) and 24 h-starved (●) rats.

chromatography is less than that in specific enzymic/fluorimetric assays, as demonstrated by the variability of these results. This was particularly the case for the synthesis rates based on the methylated amino acids, as their yields were low. For the fed state, synthesis rates calculated from the various precursors and products were generally similar for total proteins, the three individual proteins and myofibrillar 3-methylhistidine. In the starved state the synthesis rates fell in all cases. In this case there was greater variability between synthesis rates calculated from the different precursors and products for the contractile proteins. In particular, synthesis rates were lowest when calculated from the methylated amino acids, and this was particularly true for actin. The reason for this may be that the contaminating proteins included in the myosin heavy chain and actin fractions had higher synthesis rates than the major species. Tyrosine and methionine would have been derived from all the proteins present. In contrast, synthesis rates based on the methylated amino acids should be specific. Certainly, 3-methylhistidine synthesis in myofibrils indicated a rate which was consistent with its expected origins, indicating 85% from actin and 15% myosin heavy chain. Assuming synthesis rates based on the methylated amino acids to be the accurate values, then these synthesis rates in starvation, which can be assumed to be steady-state replacement rates, indicated turnover of aldolase, myosin heavy chain and actin to be in the proportions 1:0.72:0.29 (i.e. half-lives of 7.5, 10 and 26 days respectively) for rats of this age. It is apparent that myosin heavy-chain synthesis is twice as fast as that of actin, even if values based on methionine or tyrosine are used. It is also apparent that the disproportionate fall in myofibrillar protein synthesis on starvation (Bates & Millward, 1983) results from the disproportionate fall in actin synthesis.

Discussion

Turnover of protein-bound 3-methylhistidine in rat skeletal muscle

All of the measurements in these studies were of synthesis rates. These rates will only be similar to degradation rates in the adult female rats (Expt. D; Table 2) in which muscle growth has stopped and, for a limited period, in the muscle of starved growing rats after the expansion of muscle mass, after feeding, has ceased and before losses of muscle protein have commenced. Thus the equation of synthesis rates with degradation can only be made under particular circumstances. This is not always appreciated, e.g. by Harris (1981), who was unable to understand why in our previous paper (Millward *et al.*, 1980) we did not equate 3-methylhistidine degradation with synthesis rates measured in young

fed rats. Similarly, Munro & Young (1981) show results for actin and myosin synthesis in growing rats and discuss the data in terms of turnover (i.e. degradation). The present results show that in the growing rat it is especially difficult in the case of 3-methylhistidine because of the marked changes in synthesis that occur between feeding and starvation.

Indeed, even between the various groups of fed animals 3-methylhistidine synthesis rates varied markedly. Because the lowest rates were those measured during the 6 h constant infusion (Expts. A and B, Table 1), it can probably be assumed that this was because these rats did not eat during the infusion. This is supported by the fact that the highest rate of 3-methylhistidine synthesis (equal to the overall rate of protein synthesis) was observed in the most rapidly growing young rats measured immediately after feeding with access to food throughout the 60 min incorporation period.

Watkins & Morgan (1979) reported similar rates of total protein and 3-methylhistidine synthesis measured in the perfused heart. Since insulin, which stimulates protein synthesis, was present, these findings are consistent with our results of similar rates of synthesis in the fed state. Certainly Preedy (1981) reported a small but significant selective increase in myofibrillar protein synthesis in the perfused hemi-corpus compared with the rate measured *in vivo*. Clearly, given the obvious sensitivity of 3-methylhistidine synthesis to nutritional state, the attainment of true steady-state conditions is not easy. However, we believe that the measurement in the adult female rats (Expt. D; Table 2) and in the 24 h-starved young rats (Expt. E; Table 3) represent steady-state conditions, with synthesis rates similar to degradation rates. The higher steady-state synthesis rate reported here for the young rats (3.3%/day for 3-methylhistidine isolated from actomyosin) compared with our previous value for adult rats (1.08%/day; Millward *et al.*, 1980) reflects the higher overall turnover in the young rats.

In the adult female rats overall growth is very slow, but, to avoid the possibility that there was a diurnal expansion and contraction of the muscle mass, we made measurements in the fed and starved states. The similarity of the results in the two states means that we can be reasonably certain that the 3-methylhistidine synthesis rate, less than 40% of the overall rate, was the replacement or turnover rate. In young starved rats of the weight used in the current experiment (i.e. 100 g), we (Millward & Waterlow, 1978) and others (see Garlick, 1980) have made daily measurements of muscle protein mass and have not observed any losses for at least 2 days. Thus we feel confident therefore that in these rats also the synthesis rates were very similar to the replacement rates.

We also must consider whether the slower rate of 3-methylhistidine synthesis compared with the overall rate is an artifact caused by erroneous values for the precursor labelling. Thus in Expt. E (Table 3), if the labelling of tyrosine and methionine in the actual precursor pools was over twice the measured values, the overall rate of synthesis would be the same as that for 3-methylhistidine. Since the highest possible labelling is that of plasma tyrosine and methionine, and since the area under the plasma tyrosine (Fig. 3*b*) and methionine (Fig. 3*d*) curves was less than 20% greater than that for the muscle free amino acids, the maximum potential overestimation of the overall synthesis rate was less than 20%. We are less confident about the labelling of the actual *S*-adenosylmethionine responsible for histidine methylation. If the measured value was over twice the actual value, then this would account for the low rate of 3-methylhistidine synthesis compared with the overall rate. However, we believe that the results in Expt. E unequivocally demonstrate that precursor compartmentation cannot account for the lower rate of 3-methylhistidine synthesis. 3-Methylhistidine synthesis in actin was less than trimethyl-lysine synthesis in myosin, and presumably the same precursor was involved. Similarly, synthesis rates based on both tyrosine and methionine in the actin fraction were lower than the overall rate based on the same amino acids. Nevertheless the approach adopted in Expt. D (Table 2), the incorporation of labelled histidine into mixed proteins and 3-methylhistidine, was designed to prevent any problem of precursor compartmentation.

The only assumption in Expt. D (Table 2) is that methylation of histidine is an early post-translational event, since, if methylation was delayed after translation (i.e. if there was a significant pool of non-methylated contractile proteins) the time course of appearance of labelled histidine in protein-bound 3-methylhistidine would be delayed and measurement at early times would result in apparently lower synthesis rates. Morse *et al.* (1975) examined methylation of proteins in cultured chick muscle cells and found that methylation was a very early event, probably involving ribosome-bound nascent chains. Similar conclusions were reached by Miyake & Kakimoto (1976), who demonstrated similar inhibition of protein synthesis and methylation in several tissues *in vivo* after puromycin treatment. Watkins & Morgan (1979) demonstrated in the perfused heart that cycloheximide depressed protein synthesis and protein methylation to similar extents. In any case our most recent measurements in rabbits and rats (P. C. Bates, G. J. Laurent & D. J. Millward, unpublished work) show that the same low ratio of labelling of 3-methylhistidine is observed as late as 4 h after the injection. All these studies support the assumption that relative specific radio-

activities of histidine and 3-methylhistidine 60 min after histidine injection are equal to relative synthesis rates. These results demonstrate, then, that proteins containing 3-methylhistidine in the myofibril do have slower turnover rates than the overall rate for skeletal muscle.

Relative turnover rates of actin and myosin heavy chain

As discussed in the introduction to this paper, there is no consensus in the literature about the relative turnover rates of actin and myosin. A possible explanation of the varied findings is in the study of Loble & Lovie (1979), who have reported that in amino acid labelling experiments the specific radioactivity of actin varies according to the extraction procedure. Low-yield preparations gave a labelling of actin which was only 60% of that of myosin, whereas high-yield preparations gave actin which was labelled similarly to myosin. Since myosin labelling only varied slightly according to the preparation, they concluded that loss of newly synthesized highly labelled actin molecules occurred in some of their preparations (as reported by Etlinger *et al.*, 1975) and that when this was minimized similar synthesis rates of actin, myosin, actomyosin and mixed muscle proteins were indicated. Why the labelling of only actin varied with the various preparations when Etlinger *et al.* (1975) showed that highly labelled newly synthesized myosin heavy chain was also lost in some myofibrillar washing procedures was not explained. However, the major difficulty of relating the measurements of Loble & Lovie (1979) to measurements of actin and myosin turnover is that it is not possible to equate their synthesis-rate measurements with degradation, since an unspecified number of their rabbits were young growing animals, in which relative synthesis rates could have been (according to our results) closer than relative degradation rates.

In our experiments it is highly unlikely that differential losses of newly synthesized actin could account for the lower labelling of actin. This is because myofibrils were washed in a non-relaxing buffer, which, according to Etlinger *et al.* (1975), does not result in release of myofilaments. After this, the whole myofibrillar fraction was dissolved and separated by gel filtration, so that the measured labelling was representative of the various proteins. In any case, the measurements of purified actin gave similar values to those made on 3-methylhistidine isolated from the total mixed muscle protein. Losses of highly labelled protein could not occur at all in these experiments. Since the present results are in agreement with those of Zak *et al.* (1977) and Martin (1981), who showed that actin, myosin heavy chain, myosin light chains and tropomyosin all

turned over at different rates in rat heart, we can conclude that, in the rat at least, actin turnover is markedly slower than that of myosin heavy chain.

The inclusion of aldolase in this study was based on the assumption that it would turn over more rapidly than the contractile proteins. This was reported by Dreyfus *et al.* (1960), who showed exponential loss of radioactivity from aldolase, in contrast with no loss from myosin for 30 days, at which time a precipitous loss occurred. They interpreted this as indicating lifetime kinetics for myosin, but random turnover of aldolase. According to our results, aldolase turnover is 50% faster than that of myosin and 3 times faster than that of actin. However, according to Walsh *et al.* (1980), aldolase possesses multiple binding sites for the tropomyosin-troponin complex of the thin actin-containing filaments in skeletal muscle. Indeed this may be a feature of several glycolytic enzymes (Sigel & Pette, 1969); thus distinct turnover characteristics for aldolase, other glycolytic enzymes and contractile proteins would not necessarily be expected. Although the present results indicate nothing about the nature of the degradation of aldolase or the contractile protein, apart from the relative rates, since the subunit molecular weight of aldolase is similar to that of actin (i.e. about 40 000; see Scopes, 1977), it would appear that the inverse correlation of turnover rate and molecular weight reported by Dice *et al.* (1973) does not obtain for aldolase and actin. It does for actin and myosin heavy chain (mol.wt. 220 000), however.

The implication of heterogeneous turnover of individual myofibrillar proteins when it occurs is that any mechanism to account for myofibrillar turnover must include the facility for differential removal and replacement rates of individual protein subunits from the myofibril. One possible mechanism has been suggested elsewhere (Millward, 1980).

Changes in the relative synthesis rates of individual muscle proteins

The present studies have extended our previous observations that the relative synthesis rates of myofibrillar and sarcoplasmic proteins vary according to the nutritional state (Bates & Millward, 1983). It is clear that the synthesis of actin is particularly sensitive, changing by a factor of 5, compared with a factor of less than 2 for the overall muscle protein-synthesis rate between the 24 h-starved and fed states in young rats. Furthermore, with prolonged starvation the synthesis rate of actin falls to even lower values compared with other muscle proteins (Millward *et al.*, 1982). Why actin synthesis should be this sensitive is not known, but, as discussed in the preceding paper (Bates & Millward, 1983), this may be a general feature of proteins with slow steady-state turnover rates. One possible mechanism

for the differential regulation of individual protein synthesis is that demonstrated by Lodish (1974) for the regulation of initiation of translation of the two mRNA species for globin in reticulocytes. In this model it is suggested that, if the binding affinity for a mRNA and the initiation complex varies between mRNA species, then with changes in the overall rate of initiation there will be a relatively greater increase in initiation frequencies of those species of mRNA with the lowest binding affinities. In the present experiments it is not possible to determine the extent to which the fall in the rate of protein synthesis on starvation was associated with a decrease in the number of mRNA species or the decreased rates of mRNA translation, and, since there was a fall in RNA concentration and activity (results not shown), both mechanisms could have occurred. Clearly this is a problem deserving more study.

This work was generously supported by the Muscular Dystrophy Group of Great Britain and the Medical Research Council.

References

- Bates, P. C. & Millward, D. J. (1981) *Proc. Nutr. Soc.* **40**, 89A
- Bates, P. C. & Millward, D. J. (1983) *Biochem. J.* **214**, 587–592
- Bates, P. C., Millward, D. J. & Rennie, M. J. (1981) *J. Physiol. (London)* **317**, 20P
- Bates, P. C., DeCoster, T., Grimble, G. K., Holloszy, J. O. & Millward, D. J. (1980) *J. Physiol. (London)* **299**, 52P
- Bidinost, L. E. (1951) *J. Biol. Chem.* **190**, 423–430
- Brown, J. G., Bates, P. C., Holliday, M. A. & Millward, D. J. (1981) *Biochem. J.* **194**, 771–782
- Dice, J. F., Dehlinger, P. J. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 4220–4228
- Dreyfus, J. C., Kruh, J. & Schapira, G. (1960) *Biochem. J.* **75**, 574–578
- Eloranta, T. O., Kajander, E. O. & Raina, E. M. (1976) *Biochem. J.* **160**, 287–294
- Etlinger, J. D., Zak, R., Fishman, D. A. & Rabinowitz, R. (1975) *Nature (London)* **255**, 259–261
- Garlick, P. J. (1980) *Compr. Biochem.* **19B**, 77–152
- Garlick, P. J., Millward, D. J. & James, W. P. T. (1973) *Biochem. J.* **136**, 935–945
- Grimble, G. K. (1981) Ph.D. Thesis, University of London
- Harris, C. I. (1981) *Biochem. J.* **194**, 1011–1014
- Haverberg, L. N., Munro, H. N. & Young, V. R. (1974) *Biochim. Biophys. Acta* **371**, 226–237
- Huszar, G. & Elzinga, M. (1971) *Biochemistry* **10**, 229–236
- Kaufman, S. (1970) *Methods Enzymol.* **17A**, 603–609
- Klee, C. B., Crouch, T. H. & Richman, P. G. (1980) *Annu. Rev. Biochem.* **49**, 489–515
- Koizumu, T. (1974) *J. Biochem. (Tokyo)* **76**, 431–439
- Lobley, G. E. & Lovie, J. M. (1979) *Biochem. J.* **182**, 867–874
- Lodish, H. F. (1974) *Nature (London)* **251**, 385–388

- Low, R. B. & Goldberg, A. L. (1973) *J. Cell Biol.* **56**, 590–595
- Martin, A. F. (1981) *J. Biol. Chem.* **256**, 964–968
- Millward, D. J. (1980) *Compr. Biochem.* **19B**, 153–232
- Millward, D. J. & Waterlow, J. C. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 2283–2290
- Millward, D. J., Bates, P. C., Grimble, G. K., Brown, J. G., Nathan, M. & Rennie, M. J. (1980) *Biochem. J.* **190**, 225–228
- Millward, D. J., Bates, P. C., Broadbent, P. & Rennie, M. J. (1982) in *Clinical Nutrition '81* (Wesdorp, R. I. C. & Soeters, P. B., eds.), pp. 190–203, Churchill-Livingstone, Edinburgh
- Miyake, M. & Kakimoto, Y. (1976) *Metab. Clin. Exp.* **25**, 885–896
- Morse, R. K., Vergnes, J. P., Malloy, J. & McManus, J. R. (1975) *Biochemistry* **14**, 4316–4324
- Munro, H. N. & Young, V. R. (1981) in *Nitrogen Metabolism in Man* (Waterlow, J. C. & Stephen, J. M. L.), pp. 495–508, Applied Science Publishers, London
- Perry, B. N. (1974) *Br. J. Nutr.* **31**, 35–45
- Preedy, V. R. (1981) Ph.D. Thesis, University of London
- Scopes, R. K. (1977) *Biochem. J.* **161**, 253–263
- Sigel, P. & Pette, D. (1969) *J. Histochem. Cytochem.* **17**, 225–237
- Swick, R. W. & Song, H. (1974) *J. Anim. Sci.* **38**, 1150–1157
- Trayer, I. P., Harris, C. I. & Perry, S. V. (1968) *Nature (London)* **217**, 452–454
- Velick, S. F. (1956) *Biochim. Biophys. Acta* **20**, 228–236
- Walsh, T. P., Winzer, D. J., Clarke, F. M., Masters, C. J. & Morton, D. J. (1980) *Biochem. J.* **186**, 89–98
- Waterlow, J. C. & Stephen, J. M. L. (1967) *Clin. Sci.* **33**, 489–506
- Waterlow, J. C., Garlick, P. J. & Millward, D. J. (1978) *Protein Turnover in Mammalian Tissues and in the Whole Body*, Elsevier/North-Holland, Oxford
- Watkins, C. A. & Morgan, H. E. (1979) *J. Biol. Chem.* **254**, 693–701
- Zak, R., Ratkitzis, E. & Rabinowitz, M. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 1147A
- Zak, R., Martin, A. F., Prior, G. & Rabinowitz, M. (1977) *J. Biol. Chem.* **252**, 3430–3435