The effects of fasting or hypoxia on rates of protein synthesis *in vivo* in subcellular fractions of rat heart and gastrocnemius muscle

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We measured rates of protein synthesis *in vivo* in subcellular fractions (soluble, myofibrillar and stromal fractions) of the heart and the gastrocnemius from rats after fasting or under hypoxic conditions (i.e. atmospheres containing 5% or 10% O₂). Such interventions are known to inhibit protein synthesis under some circumstances. The recovery of tissue protein after fractionation was 80–100%. The proportions of protein present in the soluble and stromal fractions were different in the two muscles. The rates of protein synthesis in the myofibrillar and stromal fractions were less than those for total mixed tissue protein, whereas the rate for soluble protein was greater. Both fasting and moderate hypoxia (10% O₂ for 24 h) inhibited protein synthesis in the gastrocnemius. In this tissue, the synthesis of the myofibrillar fraction was apparently the most sensitive to inhibition, and this resulted in some significant increases in the soluble-fraction/myofibrillar-fraction protein-synthesis rate ratios. In the heart, fasting inhibited protein synthesis, but moderate hypoxia (10% O₂ for 24 h) did not. The rate of protein synthesis in the cardiac myofibrillar fraction was again more sensitive to fasting than were the rates in the other fractions, but it was not as sensitive as that in the gastrocnemius. Under severely hypoxic conditions (5% O₂ for 1 or 2 h), protein synthesis was decreased in all fractions in both tissues. These results suggest that the rates of protein synthesis in these relatively crude subcellular fractions vary.

INTRODUCTION

There have been many studies in which the effects of interventions such as fasting, hormone administration etc. on muscle total protein synthesis *in vivo* have been investigated (for a review, see [1]). There have also been several investigations of the turnover of specific proteins (particularly those of the myofibril; see, e.g., [2,3]). However, there have been relatively few studies in which the rates of protein synthesis in various subcellular fractions (soluble, myofibrillar etc.) of muscle have been investigated.

Early experiments *in vivo* suggested that the rates of synthesis of myofibrillar (and soluble) proteins were similar to those of mixed protein [4,5]. However, more recent studies showed that the myofibrillar k_s was less than that of the soluble fraction and that the k_s values were differentially inhibited by fasting [6–8]. Experiments *in vitro* have been of little help, since results have also been contradictory [6,9,10]. The reasons for some of the discrepancies may be methodological, since different methods have been used for administration of radio-labelled precursors and for preparation of the subcellular fractions. In addition, the nutritional state of the animals has differed.

We used the 'flooding dose' method [11] to incorporate [4-³H]phenylalanine into protein, followed by fractionation of heart or gastrocnemius into soluble, myofibrillar

and stromal fractions. This method is considered to be the most reliable of current methods for the measurement of k_s values *in vivo* but has not been widely applied to their measurement in subcellular fractions. We examined the effects of two interventions known to inhibit k_s *in vivo*, namely fasting [12] and hypoxia [13]. In this paper we demonstrate that protein in the various subcellular fractions is apparently synthesized at different rates and that k_s values in the different fractions are differentially affected by fasting or hypoxia.

EXPERIMENTAL

Materials and animals

Sources have been given previously [13,14]. Male rats weighed about 50 g on arrival and were allowed to grow to the appropriate body weight as described in ref. [14]. They were weighed each day to ensure that they were growing normally. When 48 h-fasted rats were used, they were housed in grid-bottomed cages for 3 days before the withdrawal of food as well as for the period during which food was withdrawn.

Induction of hypoxia *in vivo*

Experiments were commenced at 08:00–11:00 h. Rats were exposed to air or to hypoxic gas mixtures (O₂/N₂ 1:9 or 1:19) in chambers as previously described [13].

Abbreviations used: k_s , fractional rate of protein synthesis (%/day); k_{RNA} , efficiency of protein synthesis relative to RNA (g of protein synthesized/g of RNA per day); S_p , plasma free [4-³H]phenylalanine specific radioactivity; S_t , whole-tissue free [4-³H]phenylalanine specific radioactivity; S_b , protein-bound [4-³H]phenylalanine specific radioactivity; C_s , capacity for protein synthesis, i.e. RNA/protein ratio (μ g of RNA/mg of protein); n , number of observations.

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For Expt. 2, in which rats were exposed to the hypoxic atmosphere (O_2/N_2 1:9) for 24 h, food was withdrawn from the time of onset of hypoxia, but the rats had access to water. Thus, although rats were post-prandial when they were placed in the chambers, they had been fasted for 24 h at the time that measurements of k_s were made. For the shorter periods of more severe hypoxia (O_2/N_2 1:19 for 1 or 2 h, Expt. 3), 48 h-fasted rats were used.

Measurement of protein-synthesis rates

Protein synthesis was measured by the 'flooding dose' method of Garlick *et al.* [11]. For Expt. 1, in which the effects of fasting were studied, the rat's body was rapidly wrapped in a cloth in such a way that its limbs were restrained but its head and tail were free. [$4\text{-}^3\text{H}$]Phenylalanine (150 mM; sp. radioactivity 1.0 Ci/mol; 1.5 $\mu\text{mol/g}$ body wt.) was injected into a lateral tail vein, after which the rat was released. At 10 min after the injection, the rat was decapitated. A sample of blood was collected into a heparinized tube for the determination of S_p . For the subcellular-fractionation experiments, the heart and the right hind-limb were removed and immersed as quickly as possible in an ice/water mixture. The hind-limb was dissected and the gastrocnemius was removed. Tissues were blotted, weighed, frozen in liquid N_2 and stored at -80°C until they were processed. Rates of protein synthesis were determined as previously described [11,14].

For Expts. 2 and 3, in which the effects of hypoxia were studied, the rat was removed from its chamber and rapidly wrapped. Its head was placed in a cranial hood into which the appropriate gas mixture was passed. [$4\text{-}^3\text{H}$]Phenylalanine (150 mM; sp. radioactivity 0.33 Ci/mol; 1.5 $\mu\text{mol/g}$ body wt.) was injected and the rat was returned to its chamber. At 10 min after the injection, the rat was removed from its chamber and was processed as described above.

Calculation of k_s and k_{RNA} values

Values of k_s in units of %/day were calculated from the formula:

$$k_s = \frac{100 \cdot S_b}{S_i \cdot t}$$

where t is the time between injection of [$4\text{-}^3\text{H}$]phenylalanine and immersion of the tissue in ice/water. Thus, because of the time interval between decapitation and tissue cooling, the values of k_s may be slight underestimates of the true values *in vivo*. However, because of the consistency in the times of tissue immersion in ice/water (Table 1), k_s values in the same tissue should be strictly comparable. In these experiments, we used the final values of S_i for calculation of k_s . We assumed that there was no decline in S_i over the course of the experiments. Our recent investigations [15] in mature (approx. 200 g body wt.) female rats have suggested that this is justified for striated muscles. Values of k_{RNA} were calculated as described in ref. [15].

Subcellular fractionation of heart and gastrocnemius

The ventricles of the heart and the gastrocnemius were fractionated essentially as described previously [10]. All operations were carried out at $0\text{--}4^\circ\text{C}$. Tissue was homogenized in 10 ml of Buffer A [10 mM-imidazole, 60 mM-KCl, 0.5 mM-EGTA, 4 mM-MgCl₂, 1 mM-dithiothreitol, 0.5% (v/v) Triton X-100, pH 7.4] with a Polytron

homogenizer (maximum power setting). At this stage, a sample (0.33 vol.) of homogenate was removed for the determination of the mixed-protein k_s . The homogenate volume was made up to 40 ml with Buffer A and was centrifuged at 33000 g for 10 min. The supernatant fraction (1st soluble fraction) was retained. The precipitate was re-extracted with Buffer A (40 ml) in a ground-glass homogenizer and re-centrifuged as above. The supernatant fraction (2nd soluble fraction) was retained. The precipitate was extracted with 7 ml of Buffer B (100 mM-KH₂PO₄, 50 mM-K₂HPO₄, 300 mM-KCl, 1 mM-EDTA, 5 mM-ATP, pH 6.3) in a ground-glass homogenizer. The suspension was centrifuged at 33000 g for 10 min. The precipitate was similarly re-extracted twice with Buffer B, and the three supernatants were combined. Protein insoluble in Buffer B was extracted with 10 ml of 0.3 M-NaOH (stromal fraction). The myofibrillar protein solubilized by extraction in Buffer B was re-precipitated by dilution with 6 vol. of ice-cold water with stirring and was collected by centrifugation at 33000 g for 10 min. The myofibrillar protein was dissolved in 10 ml of 0.3 M-NaOH. Protein from all four fractions (1st and 2nd soluble, myofibrillar and stromal) was precipitated by addition of 6 M-trichloroacetic acid to a final concentration of 0.6 M. Precipitated protein was washed extensively (eight washes) with 0.25 M-HClO₄ (10 ml) to remove free [$4\text{-}^3\text{H}$]phenylalanine before determination of k_s .

Other methods

Protein was determined as described in either ref. [16] or [17], depending on the sensitivity required, with bovine serum albumin as standard. RNA was determined from the A_{232} and A_{260} of HClO₄ extracts after alkaline hydrolysis of RNA [18]. Statistical significance was determined by analysis of variance, with values of $P < 0.05$ being taken to be statistically significant.

RESULTS

Recoveries of protein

Data for individual fractions are given in Table 2. The empirical values were adjusted for the sample of homogenate removed for the determination of the mixed-protein k_s (see the Experimental section). Recoveries of protein were always greater than 82% and were similar for a given tissue when the effect of a given intervention was studied (Table 2). The proportional distribution of protein can be calculated from these data. The myofibrillar contents of the heart and gastrocnemius were similar (about 17%). Recovery of the myofibrillar fraction is likely to be the lowest of all three fractions, because its preparation requires most manipulation. The values for myofibrillar protein content are likely to be an underestimate. We have previously shown that the myofibrillar component accounted for about 30% of the total protein in the ventricles of hearts of 330 g rats, given that its recovery by the procedure described was about 75% [10]. When this correction factor is used, the myofibrillar fraction constituted about 23% of cardiac protein. Recovery of the myofibrillar fraction may be less from the smaller hearts used in the present experiments, merely because of the technical problems of handling smaller quantities of protein. Alternatively, the myofibrillar fraction may indeed constitute a smaller proportion of total heart protein in the immature animals

Table 1. Times of immersion of tissues in ice/water and specific radioactivities of free and protein-bound [4-³H]phenylalanine in plasma and tissues

Rats were killed and tissues were processed as described in the Experimental section. For Expt. 1, the body weight of the group of fed rats was 90.3 ± 1.5 g ($n = 8$) at the time k_s was measured. The initial body weight of the group of rats destined for fasting was 85.3 ± 1.8 g ($n = 7$) at the time of removal of food. Body weight decreased to 62.3 ± 1.7 g after 48 h of fasting. For Expt. 2, all rats were fed when exposed to 24 h of normoxia (body wt. 131.8 ± 0.9 g, $n = 8$) or 24 h of hypoxia (10% O₂, body wt. 130.4 ± 1.5 g, $n = 9$). They did not have access to food during the experimental period, but were in the fed state at the time they were placed in the chambers for exposure to normoxia or hypoxia. For Expt. 3, all rats were fasted for 48 h before the normoxic experimental period (body wt. 97.9 ± 1.4 g, $n = 5$), or before exposure to 5% O₂ for 1 h (body wt. 98.0 ± 1.0 g, $n = 4$) or 5% O₂ for 2 h (body wt. 96.3 ± 1.4 g, $n = 4$). Statistical significance of results is as follows: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ for the 48 h-fasted group versus the fed group in Expt. 1 or for the hypoxic groups versus the normoxic groups in Expts. 2 and 3.

Tissue	Condition	Time of immersion (min)	S_p (d.p.m./nmol)	S_i (d.p.m./nmol)	S_o of mixed protein (d.p.m./nmol)	S_o of myofibrillar protein (d.p.m./nmol)	S_o of soluble protein (d.p.m./nmol)	S_o of stromal protein (d.p.m./nmol)	Soluble S_o /myofibrillar S_o
Expt. 1 Heart	Fed	11.78 ± 0.05	2079 ± 22	1786 ± 19	2.553 ± 0.131	1.902 ± 0.100	2.676 ± 0.086	2.273 ± 0.109	1.443 ± 0.075
	48 h fasted	11.92 ± 0.05	2259 ± 33	1887 ± 54	0.997 ± 0.125 ^c	0.597 ± 0.110 ^c	1.215 ± 0.152 ^c	0.947 ± 0.121 ^c	1.915 ± 0.190 ^a
Gastrocnemius	Fed	10.71 ± 0.04	2066 ± 20	1880 ± 22	1.952 ± 0.082	1.484 ± 0.050	2.354 ± 0.100	1.663 ± 0.063	1.586 ± 0.028
	48 h fasted	10.71 ± 0.04	2259 ± 33	2005 ± 73	0.439 ± 0.040 ^c	0.182 ± 0.023 ^c	0.706 ± 0.076 ^c	0.311 ± 0.027 ^c	3.740 ± 0.268 ^c
Expt. 2: 24 h-fasted rats Heart	24 h normoxia	11.81 ± 0.04	757 ± 6	727 ± 8	0.406 ± 0.018	0.294 ± 0.019	0.483 ± 0.020	0.248 ± 0.012	1.679 ± 0.090
	24 h hypoxia (10% O ₂)	11.82 ± 0.04	743 ± 6	730 ± 9	0.399 ± 0.017	0.334 ± 0.023	0.502 ± 0.015	0.258 ± 0.017	1.537 ± 0.082
Gastrocnemius	24 h normoxia	10.56 ± 0.03	757 ± 6	731 ± 6	0.277 ± 0.010	0.206 ± 0.011	0.362 ± 0.012	0.211 ± 0.019	1.753 ± 0.041
	24 h hypoxia (10% O ₂)	10.55 ± 0.01	743 ± 6	721 ± 8	0.191 ± 0.017 ^b	0.120 ± 0.009 ^c	0.258 ± 0.015 ^c	0.140 ± 0.012 ^b	2.182 ± 0.064 ^c
Expt. 3: 48 h-fasted rats Heart	Normoxia	11.66 ± 0.03	761 ± 9	736 ± 18	0.279 ± 0.037	0.221 ± 0.028	0.374 ± 0.035	0.170 ± 0.032	1.770 ± 0.185
	1 h hypoxia (5% O ₂)	11.81 ± 0.05	748 ± 12	749 ± 6	0.141 ± 0.019 ^b	0.117 ± 0.017 ^b	0.183 ± 0.022 ^c	0.096 ± 0.022 ^a	1.597 ± 0.112
	2 h hypoxia (5% O ₂)	11.79 ± 0.06	726 ± 5	723 ± 15	0.115 ± 0.027 ^c	0.094 ± 0.016 ^b	0.154 ± 0.031 ^c	0.062 ± 0.011 ^b	1.601 ± 0.185
Gastrocnemius	Normoxia	10.58 ± 0.02	761 ± 9	741 ± 6	0.175 ± 0.033	0.108 ± 0.029	0.247 ± 0.030	0.126 ± 0.028	2.593 ± 0.328
	1 h hypoxia (5% O ₂)	10.63 ± 0.01	748 ± 12	714 ± 14	0.055 ± 0.008 ^c	0.034 ± 0.006 ^c	0.073 ± 0.012 ^c	0.038 ± 0.005 ^b	2.146 ± 0.059
	2 h hypoxia (5% O ₂)	10.56 ± 0.01	726 ± 5	686 ± 15	0.081 ± 0.025 ^b	0.063 ± 0.024	0.113 ± 0.035 ^c	0.067 ± 0.020	1.995 ± 0.216

Table 2. Recovery of protein in subcellular fractions from the heart and gastrocnemius

Tissues were processed as described in the Experimental section. Details of the rats used are given in the legend to Table 1. The values of protein recovered in the 1st and 2nd soluble fractions were combined. Statistical significance of results is as follows: ^a*P* < 0.05, ^b*P* < 0.01, ^c*P* < 0.001 for the 48 h-fasted group versus the fed group in Expt. 1 or for the hypoxic groups versus the normoxic groups in Expts. 2 and 3.

Tissue	Condition	Total tissue protein (mg)	Myofibrillar-fraction protein (mg)	Soluble-fraction protein (mg)	Stromal-fraction protein (mg)	Recovery (%)
Expt. 1						
Heart	Fed	42.51 ± 1.07	7.14 ± 0.24	24.25 ± 1.30	5.66 ± 0.33	87
	48 h fasted	33.22 ± 1.46 ^c	5.74 ± 0.33 ^b	19.60 ± 0.77 ^b	4.16 ± 0.26 ^b	89
Gastrocnemius	Fed	96.33 ± 2.57	16.51 ± 0.83	34.49 ± 0.97	28.27 ± 0.96	82
	48 h fasted	90.17 ± 3.38	17.07 ± 0.76	30.37 ± 1.56 ^a	28.23 ± 1.41	84
Expt. 2: 24 h-fasted rats						
Heart	24 h normoxia	50.75 ± 1.39	10.79 ± 0.36	32.67 ± 1.03	7.55 ± 0.37	100
	24 h hypoxia (10% O ₂)	56.92 ± 2.05 ^b	12.11 ± 0.49 ^a	37.92 ± 1.12 ^b	7.84 ± 0.20	100
Gastrocnemius	24 h normoxia	159.8 ± 3.0	43.46 ± 1.78	53.16 ± 1.41	40.73 ± 0.64	86
	24 h hypoxia (10% O ₂)	159.4 ± 4.0	40.16 ± 2.56	52.83 ± 1.74	39.88 ± 1.34	83
Expt. 3: 48 h-fasted rats						
Heart	Normoxia	46.12 ± 1.86	10.34 ± 0.52	30.62 ± 1.31	6.77 ± 0.56	100
	1 h hypoxia (5% O ₂)	45.71 ± 1.92	9.62 ± 0.71	30.52 ± 2.12	7.55 ± 0.61	100
	2 h hypoxia (5% O ₂)	45.46 ± 0.82	10.04 ± 0.32	31.20 ± 0.92	6.43 ± 0.32	100
Gastrocnemius	Normoxia	144.0 ± 4.0	43.79 ± 2.07	53.63 ± 1.56	43.10 ± 2.48	98
	1 h hypoxia (5% O ₂)	146.1 ± 5.7	44.10 ± 1.31	51.57 ± 1.39	41.57 ± 3.04	94
	2 h hypoxia (5% O ₂)	140.6 ± 6.5	45.51 ± 1.02	50.84 ± 1.33	38.46 ± 1.32	96

used in these experiments. The major differences between the heart and gastrocnemius were in the proportions of protein constituting the soluble (larger in the heart) and stromal (larger in the gastrocnemius) fractions. The homogenization conditions (high-speed blending with sonication in the presence of detergent) should have been sufficiently violent to disrupt membranes and organelles. These data emphasize that different muscles may vary considerably in the proportions of protein in each fraction, even when unsophisticated fractionation procedures such as ours are used. We did not characterize the proteins constituting the stromal fraction. It would have contained some proteins insoluble in NaOH such as collagen, which would not have reacted in the protein assays used. It has been shown that the proteins of the stromal fraction which were insoluble in NaOH did not incorporate [4-³H]phenylalanine under the experimental conditions used here, and furthermore the phenylalanine content of collagen is low (about 1%). It is thus unlikely that collagen could have contributed greatly to our measurements of *k_s*. It could be argued that the stromal fraction contained undissolved myofibrils. However, the fraction had been previously extracted three times under conditions that should have solubilized myofibrils.

Protein contents of tissues and subcellular fractions

Results are shown in Table 2. From the body weights of rats used in Expt. 1 (Table 1), it would be expected that the tissue weights of the 48 h-fasted group would be

5.5% less than those of the fed group at the time when food was removed. For the gastrocnemius, the total protein content in the fasted group was 6.4% less than in the fed group, indicating that little protein was lost from this muscle. However, for the heart, the total protein content in the fasted group was 21.9% less than in the fed group, emphasizing that the cardiac protein pool is more sensitive to short-term fasting than is the skeletal-muscle protein pool (see also [15,19,20]). This difference was also reflected in the subcellular fractions. In the subcellular fractions from the gastrocnemius, a significant loss of protein (12.0%) was detected only in the soluble fraction.

In Expt. 2, the initial body weights in the two groups of rats were closely matched (Table 1). After 24 h of hypoxia (10% O₂), heart protein mass has increased by 12.2%. This presumably represents the initiation of the hypertrophic response seen under hypoxic conditions (see below). The protein contents of the myofibrillar and soluble fractions increased significantly, by 12.2 and 16.1% respectively. There were no significant increases in other cardiac or gastrocnemius fractions. In Expt. 3, the durations of hypoxia were too short to cause any detectable alterations in protein contents.

Rates of protein synthesis in mixed protein and in subcellular fractions of heart and gastrocnemius from fasted rats

Rates of protein synthesis were calculated from *S₀* and

S_i values measured in tissues of rats decapitated at 10 min, i.e. it was assumed that equilibration of S_p with the specific radioactivity of [4- 3 H]phenylalanyl-tRNA was rapid and that the specific radioactivity of the [4- 3 H]-phenylalanyl-tRNA did not decline significantly over the 10 min after the injection of [4- 3 H]phenylalanine. Previous work has shown this work to be justified in striated muscles [11,15]. Values of S_p , S_i and S_b are shown in Table 1, as are the times after injection of the [4- 3 H]-phenylalanine at which tissues were immersed in ice/water. In Expt. 1, S_i values were $> 84\%$ of S_p values. In Expts. 2 and 3, equilibration was complete. The times at which hearts were immersed in ice/water were similar (Table 1), as were the times at which gastrocnemius muscles were immersed. The interval between injection of [4- 3 H]phenylalanine and immersion was some 10% greater for the heart than for the gastrocnemius. Thus it would be imprudent to compare k_s values directly between tissues when the differences are small.

Effects of 48 h of fasting on k_s of mixed protein and k_s in the subcellular fractions were studied in Expt. 1 (Table 3). The k_s values of the 2nd soluble fraction are not shown, since they were little different from those of the 1st soluble fraction. Fasting decreased cardiac and gastrocnemius mixed-protein k_s by 64 and 79% respectively. These changes resulted from decreases in cardiac C_s and k_{RNA} of 25 and 52% respectively, and decreases in gastrocnemius C_s and k_{RNA} of 32 and 69% respectively. These experiments confirm our previous observations that decreases in cardiac or gastrocnemius k_s during fasting result from changes in both C_s and k_{RNA} [15].

Myofibrillar k_s values were less than those for mixed protein in fed or fasted rats by 24–40%. In the gastrocnemius, the k_s for the soluble fraction was significantly greater than, and that for the stromal fraction was significantly less than, the k_s for mixed protein. In the heart the pattern was similar, although the differences were not significantly different. It could be argued that these differences could have resulted from there being different precursor pools of [4- 3 H]-phenylalanine of different specific radioactivities for the synthesis of the various fractions, although, in view of the method used, we consider this unlikely. Values of mixed-protein k_s were calculated from the k_s value of each fraction and the proportion of protein recovered in that fraction. Calculated k_s values agreed well with empirical k_s values for mixed protein (Table 3).

The S_b and k_s values in all three subcellular fractions from both tissues decreased on fasting (Tables 1 and 3). However, the decreases were proportionately greater in the myofibrillar fractions than in the others. Hence, the cardiac soluble-protein/myofibrillar-protein S_b ratio was significantly increased, by 33% (Table 1). In the gastrocnemius, the soluble-protein/myofibrillar-protein S_b ratio was increased by 136% by fasting.

Rates of protein synthesis in mixed protein and subcellular fractions of the heart and gastrocnemius from rats exposed to hypoxic atmospheres

We have previously shown that exposure of rats *in vivo* to atmospheres containing 10% O_2 for 6 h significantly decreased cardiac mixed-protein k_s by 20% and decreased gastrocnemius mixed-protein k_s (albeit not significantly) by 17% [13]. The decreases were entirely attributable to decreases in k_{RNA} . In Expts. 2 and 3 (Table 3), we

examined whether more prolonged (24 h, 10% O_2) or more severe (5% O_2 , 1 or 2 h) hypoxia exacerbated the inhibition of k_s and whether any of the subcellular fractions were specifically more affected.

Calculated k_s values for mixed protein were in good agreement with experimentally determined values. In the heart, mixed-protein k_s was not significantly different from values for the normoxic group after 24 h of exposure to 10% O_2 (Table 3, Expt. 2). Thus protein synthesis in the heart recovered from its initial inhibition after 6 h of hypoxia [13]. A comparison of k_s values between the three subcellular fractions of the normoxic and hypoxic groups did not show any significant differences under the two conditions. There were differences between the k_s values of the subcellular fractions and that of mixed protein. In the normoxic group, the myofibrillar k_s was significantly less than that of mixed protein (cf. Expt. 1). In Expt. 2, the k_s values for the cardiac soluble fractions were significantly greater than, and those for the stromal fractions were significantly less than, the k_s for mixed protein. As would be expected, there were no significant differences between the soluble/myofibrillar S_b ratios for the various cardiac fractions (Table 1).

Exposure of rats to an hypoxic atmosphere causes cardiac hypertrophy. Although it is the right ventricle that is primarily affected (because of the increase in pulmonary arterial pressure associated with pulmonary vasoconstriction), there is also hypertrophy of the left ventricle [21,22], possibly because of increased cardiac output and/or heart rate [23]. The recovery of cardiac k_s to control values after 24 h of hypoxia (compared with the inhibition that we observed after 6 h of hypoxia [13]) may indicate that the protein-synthesis machinery is adapting to an increased pressure-volume workload. The responses of cardiac k_s to 6 h or 24 h of hypoxia were apparently mediated by changes in k_{RNA} in the absence of any detectable changes in C_s ([13] and Table 3).

The situation in the gastrocnemius is very different from that in the heart. After 24 h of hypoxia (10% O_2), mixed-protein k_s was significantly decreased, by 30%. Protein synthesis was significantly inhibited in all subcellular fractions. The myofibrillar-fraction S_b and k_s were disproportionately more inhibited by hypoxia (Expt. 2, Tables 1 and 3). Hypoxia significantly decreased gastrocnemius k_{RNA} , but did not alter C_s . The k_s values in the myofibrillar and stromal fractions were less than, and those in the soluble fractions were greater than, those for mixed proteins.

In Expt. 3, the effects of short-term severe hypoxia were studied in 48 h-fasted rats. (Rats were fasted in order to obviate differences in food absorption caused by the inhibition of gastric emptying by hypoxia [13].) The k_s values were decreased in all fractions, sometimes by as much as 69% (Table 3). The pattern of subcellular-fraction k_s was similar to that described in Expts. 1 and 2 (Table 3). These results emphasize that hypoxia can rapidly result in inhibition of k_s .

It could be argued that we did not see effects of hypoxia on cardiac k_s in Expt. 2 (Table 3) because protein synthesis is already maximally inhibited in these post-absorptive rats (which had been fasted for 24 h at the time that k_s was measured). We do not think that this is the case, since cardiac k_s is clearly capable of further inhibition by short-term (albeit severe) hypoxia in 48 h-fasted rats. Furthermore, in Expt. 2, we demonstrated that gastrocnemius k_s was inhibited by 24 h of hypoxia.

Table 3. Rates, capacities and efficiencies of protein synthesis in tissues

Tissues were processed as described in the Experimental section. Details of the rat groups are given in the legend to Table 1. Values of k_{RNA} refer to those for mixed protein. Statistical significance of results is as follows: ^a $P < 0.05$, ^b $P < 0.01$, ^c $P < 0.001$ for the 48 h-fasted group versus the same fraction in the fed group in Expt. 1 or for the hypoxic groups versus the normoxic groups in Expts. 2 and 3; ^d $P < 0.05$, ^e $P < 0.01$, ^f $P < 0.001$ for the k_s of a given subcellular fraction versus the k_s of mixed protein in a given tissue for a given intervention.

Tissue	Condition	k_s of mixed protein (%/day)	k_s of myofibrillar protein (%/day)	k_s of soluble protein (%/day)	k_s of stromal protein (%/day)	C_s (μg of RNA/mg of mixed protein)	k_{RNA} (g of protein/g RNA per day)	Calculated k_s of mixed protein (%/day)
Expt. 1 Heart	Fed	17.51 $\pm$ 0.99	13.02 $\pm$ 0.68 ^e	18.31 $\pm$ 0.55	15.55 $\pm$ 0.71	18.00 $\pm$ 0.56	9.72 $\pm$ 0.43	16.87
	48 h fasted	6.34 $\pm$ 0.70 ^c	3.88 $\pm$ 0.66 ^{c,d}	7.67 $\pm$ 0.89 ^c	6.06 $\pm$ 0.75 ^c	13.59 $\pm$ 0.24 ^c	4.65 $\pm$ 0.48 ^c	6.70
	Fed	14.01 $\pm$ 0.76	10.65 $\pm$ 0.46 ^e	16.92 $\pm$ 0.92 ^d	11.94 $\pm$ 0.58 ^d	11.63 $\pm$ 0.55	11.99 $\pm$ 0.43	13.84
	48 h fasted	2.94 $\pm$ 0.20 ^c	1.24 $\pm$ 0.14 ^{c,f}	4.73 $\pm$ 0.43 ^{c,e}	2.10 $\pm$ 0.16 ^{c,e}	7.87 $\pm$ 0.24 ^c	3.76 $\pm$ 0.29 ^c	2.96
Expt. 2: 24 h-fasted rats Heart	24 h normoxia	6.82 $\pm$ 0.32	4.92 $\pm$ 0.30 ^f	8.11 $\pm$ 0.35 ^d	4.17 $\pm$ 0.20 ^f	16.26 $\pm$ 0.23	4.20 $\pm$ 0.20	7.25
	24 h hypoxia (10% O ₂)	6.68 $\pm$ 0.32	5.60 $\pm$ 0.41	8.39 $\pm$ 0.29 ^c	4.33 $\pm$ 0.32 ^f	16.47 $\pm$ 0.29	4.06 $\pm$ 0.18	7.28
	24 h normoxia	5.18 $\pm$ 0.19	3.86 $\pm$ 0.21 ^e	6.76 $\pm$ 0.23 ^f	3.95 $\pm$ 0.34 ^c	8.70 $\pm$ 0.18	6.01 $\pm$ 0.21	5.01
	24 h hypoxia (10% O ₂)	3.63 $\pm$ 0.33 ^b	2.27 $\pm$ 0.18 ^{c,e}	4.90 $\pm$ 0.31 ^{c,d}	2.66 $\pm$ 0.24 ^{b,d}	8.49 $\pm$ 0.12	4.27 $\pm$ 0.38 ^b	3.44
Expt. 3: 48 h-fasted rats Heart	Normoxia	4.62 $\pm$ 0.49	3.68 $\pm$ 0.45	6.27 $\pm$ 0.51	2.84 $\pm$ 0.52 ^d	13.59 $\pm$ 0.15	3.41 $\pm$ 0.37	5.22
	1 h hypoxia (5% O ₂)	2.31 $\pm$ 0.34 ^b	1.91 $\pm$ 0.28 ^b	2.99 $\pm$ 0.37 ^e	1.57 $\pm$ 0.35 ^e	13.70 $\pm$ 0.51	1.72 $\pm$ 0.29 ^b	2.55
	2 h hypoxia (5% O ₂)	1.95 $\pm$ 0.48 ^c	1.61 $\pm$ 0.29 ^b	2.62 $\pm$ 0.56 ^e	1.05 $\pm$ 0.21 ^b	13.83 $\pm$ 0.26	1.39 $\pm$ 0.33 ^c	2.19
	Normoxia	3.22 $\pm$ 0.60	1.97 $\pm$ 0.52	4.52 $\pm$ 0.54	2.32 $\pm$ 0.51	6.94 $\pm$ 0.06	4.62 $\pm$ 0.84	3.05
Gastrocnemius	1 h hypoxia (5% O ₂)	1.04 $\pm$ 0.14 ^b	0.65 $\pm$ 0.11 ^a	1.39 $\pm$ 0.22 ^c	0.72 $\pm$ 0.09 ^b	6.85 $\pm$ 0.28	1.51 $\pm$ 0.17 ^c	0.95
	2 h hypoxia (5% O ₂)	1.63 $\pm$ 0.55 ^a	1.28 $\pm$ 0.51	2.30 $\pm$ 0.78 ^b	1.36 $\pm$ 0.43	6.72 $\pm$ 0.31	2.36 $\pm$ 0.76 ^b	1.69

DISCUSSION

General methodological considerations

At the start of this Discussion, it should be emphasized that the term 'sarcoplasmic fraction' as used by others undertaking similar investigations is probably a misnomer. Conditions of homogenization have often been sufficiently extreme (sonication in the presence of detergent) to ensure disruption of organelles such as mitochondria, from which matrix (and other) proteins would be released. Here, we have elected to rename the 'sarcoplasmic fraction' the 'soluble fraction'. We used a subcellular-fractionation procedure that was largely similar to those used by other workers who have measured k_s *in vivo* in subcellular fractions of skeletal muscle (see, e.g., [7]) so that our results could be compared with some confidence. The cardiac myofibrillar fraction has been previously characterized by SDS/polyacrylamide-gel electrophoresis [10].

We measured k_s by the 'flooding dose' method [11]. We consider this to be the most reliable of currently available methods for the measurement of k_s *in vivo*. We do not believe that this technique has been used widely to measure k_s in subcellular fractions. Other workers have used constant infusion or pulse administration of tracer quantities of radiolabelled amino acid [3–8]. The 'flooding dose' method avoids the problems of slow and incomplete equilibration between S_p and S_i . Furthermore, immobilization during constant infusion itself decreased k_s in some tissues [24]. A comparison of results obtained by the 'flooding dose' and 'constant infusion' techniques showed that the former gave consistently higher k_s values [25].

Synthesis of functional myofibrils can be divided into the synthesis of the various contractile proteins and their subsequent assembly into contractile elements. Until they are assembled into myofibrils or other polymeric species, contractile proteins would presumably fractionate with the soluble proteins. The labelling time used here was relatively short (10 min), and (as pointed out by Loble & Lovie [4]) even in fed animals may not be very much longer than the time taken to synthesize a myosin heavy chain [26]. If the time course of the precursor-product relationship for myofibrillar assembly is relatively prolonged, the apparent k_s of the myofibrillar fraction after 10 min may be less than the real k_s . The k_s values may only approach one another at longer incubation times.

Of relevance to this point is the work of Etlinger *et al.* [27], who showed that myofibrils prepared from skeletal-muscle or cardiac homogenates by extraction in a pyrophosphate/EGTA buffer contained bundles of contractile proteins which were easily dissociated from the bulk myofibrillar fraction and which remained in the supernatant fraction after centrifugation of the homogenate at 800 *g* for 10 min. Aggregation of myofilaments into these bundles could be prevented by treating the post-myofibrillar (800 *g*) supernatant with an ATP/Mg²⁺-containing 'relaxing buffer'. These bundles were presumably nascent myofibrils ('mini-filaments'). After injection of [³H]leucine, the myosin heavy-chain specific radioactivity at between 5 and 24 h after injection was higher when the source of the myosin was the 'bundles' or 'unaggregated myofilaments' rather than the 'bulk' myofibrillar fraction. At longer exposure times, the differences in heavy-chain specific radio-

activity were less. The interpretation was that the 'soluble bundles' contained newly synthesized contractile proteins which had not yet been tightly incorporated into the contractile apparatus. Thus it was suggested that misleading k_s values in the various subcellular fractions (especially the myofibrillar fraction) might result from preferential extraction of newly synthesized contractile proteins.

These possibilities were considered in detail by Bates & Millward [7] and were dismissed for three reasons. First, the conditions that they used for purification of the myofibrillar fraction were considered to be unlikely to allow extraction of newly synthesized myofilaments into the soluble fraction. Second, they showed that the ratio of soluble-protein S_b to myofibrillar-protein S_b was constant at times ranging from 10 min to 6 h of exposure to [³H]tyrosine. Third, the rates of synthesis of actin as measured by *N*-methylhistidine incorporation into total mixed protein were less than for the mixed protein itself [8]. Additionally, we have shown that after 2 h of protein synthesis the myofibrillar k_s was significantly less than that of total protein in the perfused rat heart [10]. These findings suggest that the measurements of k_s after relatively short incubation times may still be valid.

However, the possibility that there is preferential extraction of newly synthesized myofibrillar elements into the soluble fraction will probably always remain as a criticism of this type of subcellular-fractionation approach. Furthermore, it could also be argued that a similar criticism applies to the results for the stromal fraction, because k_s is also less than that of mixed protein on occasion (Table 3). Since there may always be some endogenous ATP present, it may be difficult to ensure that muscles are extracted under 'non-relaxing' conditions. Thus, since EGTA was present in our homogenization medium, it remains possible that there may be preferential solubilization of newly synthesized myofilaments even though we centrifuged at much higher speed than did Etlinger *et al.* [27]. Bates & Millward [7] did not have EGTA in their homogenization medium, but there may still have been endogenous ATP present. However, Etlinger *et al.* [27] imply that, in order for the newly synthesized myofilaments to remain in the soluble fraction after centrifugation at 10000 *g* for 10 min, it is necessary to add exogenous ATP. Neither we nor Bates & Millward [7] did this. It should also be noted that there could additionally be losses of newly synthesized myofibrillar elements at the stage of precipitation of myofibrils by dilution of the high-ionic-strength myofibrillar extract.

The considerations concerning the time taken to synthesize the myosin heavy chain compared with the labelling period may be of relevance to our findings that the k_s of the myofibrillar fraction is the most sensitive to fasting or hypoxia. As the rate of translation is progressively inhibited, the abnormally low myofibrillar k_s may be a function of the short period of labelling and the time taken to synthesize and process the larger proteins into mature myofibrils. It should be noted, however, that Preedy & Garlick [6] have also shown the plantaris myofibrillar k_s to be more sensitive to 1 day of fasting than is that of the soluble fraction, using constant infusion of [¹⁴C]tyrosine over 3 h. The inhibition was not as great as in our studies, but then the decrease in mixed-protein k_s was rather less.

Rates of protein synthesis in subcellular fractions

In Expt. 1, we sought to answer two questions: first, do different subcellular fractions have different k_s values, and, second, is there differential sensitivity of protein synthesis in the fractions to fasting? With respect to the first question, we conclude that the myofibrillar k_s is less than that of mixed protein in both heart and gastrocnemius. We could not fractionate the myofibrillar fraction further because we were limited by the relatively low amount of radioactivity incorporated during a 'flooding dose' experiment. The issue of whether protein is synthesized at different rates in subcellular fractions is contentious. Results obtained from experiments *in vitro* have differed. In the perfused heart, Sender & Garlick [9] showed that the myofibrillar-fraction protein was synthesized at the same rate as mixed and soluble protein, whereas we have shown that the myofibrillar k_s was less than that of mixed protein [10]. In the perfused hemicorpus, the k_s values of mixed, soluble and myofibrillar fractions of muscles were similar [6].

The results from experiments *in vivo* are equally complex. Using rabbits of very disparate ages and constant infusion of [^3H]tyrosine over 5–6 h, Lobley & Lovie [4] could not find any difference in k_s values of mixed, myofibrillar and soluble protein in rabbit skeletal muscle. Similar results were obtained by Laurent *et al.* [5] in the posterior and anterior latissimus dorsi muscles, heart and gizzard, using constant infusion of [^3H]proline into mature cockerels over about 6 h. Preedy & Garlick [6] showed, using constant infusion of [^{14}C]tyrosine, that in rat quadriceps or plantaris muscles the myofibrillar k_s was less than that of the soluble fraction, but a comparison with the whole-tissue k_s values was difficult to interpret. The differences in the k_s values of the soluble and myofibrillar fractions were confirmed by Bates & Millward [7]. However, it was pointed out that the values of the ratio of soluble-protein S_b to myofibrillar-protein S_b depended on the age and nutritional state of the rats. In probably the most complete previous study, Bates *et al.* [8] examined synthesis of the following in gastrocnemius and quadriceps muscles of the rat, using constant infusion or a single administration of a tracer quantity of radiolabel: mixed protein, protein-bound N^7 -methylhistidine, protein-bound trimethyl-lysine, actin, myosin and actomyosin. Protein-bound N^7 -methylhistidine synthesis was taken to be an index of actin synthesis, since even in white muscle (in which the myosin heavy chain contains N^7 -methylhistidine) actin constitutes the major protein-bound N^7 -methylhistidine pool. Protein-bound trimethyl-lysine synthesis was taken to be an index of myosin synthesis. When the time of labelling was short (1 h) and the rats were young (100 g) fed males, they found no difference in k_s in the various fractions. However, after an overnight fast, k_s values for actin and actomyosin were less than for mixed protein. It was concluded that the differences were the result of actin synthesis being very sensitive to fasting. When young fed male rats were constantly infused with radiolabel for about 6 h, the k_s values for actin (primarily) were less than for mixed proteins. This was attributed to the rats becoming post-absorptive during the infusion period. In mature non-growing female rats which were either fed or fasted for 24 h and which were pulse-labelled for 1 h, k_s for actin was less than for mixed protein.

Our results do not agree with refs. [4,5] but are in

broad agreement with refs. [6–8]. We used young fed or 24–48 h-fasted male rats. Unlike Bates *et al.* [8], we found that the myofibrillar k_s was less than the mixed-protein k_s in both the fed and the fasted groups, although the differences were proportionately greater in the fasted group (Tables 1 and 3). Labelling was always of a shorter duration than that used in [8], and this would be expected to avoid problems of animals becoming post-absorptive during the experiments. We do not understand the differences between our results and those of other workers [4,5], unless they are related to species differences or to our use of the 'flooding dose' technique or to differences in the methods of subcellular fractionation. We should also point out that all previous investigations have, to the best of our knowledge, ignored the contribution of the stromal fraction. In fact, Bates & Millward [7] state that, in several skeletal muscles, the soluble and myofibrillar fractions represent most, if not all, of the non-collagenous protein. Although we used similar techniques to Bates & Millward [7] for the preparation of the myofibrillar fractions, we always found significant proportions of protein in the stromal fractions (Table 2).

There is less of a controversy with regard to the second question. Thus, in both the heart and gastrocnemius, the myofibrillar-fraction k_s is very sensitive to inhibition by fasting compared with that of mixed protein (Tables 1 and 3). Furthermore, in both tissues fasting increased the soluble/myofibrillar S_b ratio. We have expressed results in the form of this ratio (Table 1) so that they can be directly compared with those of Preedy & Garlick [6] or Bates & Millward [7], who have also shown that the myofibrillar-fraction k_s is much more sensitive to fasting than is that of the soluble fraction of the plantaris or gastrocnemius plus quadriceps muscles of rats of various ages. Our results confirm and extend these observations. Thus the results of the present study suggest that there may be independent regulation of the synthesis of contractile and non-contractile proteins in skeletal muscle and heart.

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