Protein synthesis in skeletal muscle of the perfused rat hemicorpus compared with rates in the intact animal

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1. The rate of protein synthesis was measured in muscles of the perfused rat hemicorpus, and values were compared with rates obtained in whole animals. 2. In gastrocnemius muscle of fed rats the rate of synthesis measured in the hemicorpus was the same as that in the whole animal. However, in plantaris, quadriceps and soleus muscles rates were higher in the hemicorpus than those *in vivo*. 3. In the hemicorpus, starvation for 1 day decreased the rate of protein synthesis in gastrocnemius and plantaris muscles, in parallel with decreases in the RNA content, but the soleus remained unaffected. Similar effects of starvation were observed *in vivo*, so that the relationships between rates *in vivo* and in the hemicorpus were the same as those in fed rats. 4. Proteins of quadriceps and plantaris muscles were separated into sarcoplasmic and myofibrillar fractions. The rate of synthesis in the sarcoplasmic fraction of the hemicorpus from fed rats was similar to that *in vivo*, but synthesis in the myofibrillar fraction was greater. In the plantaris of starved rats the rates of synthesis in both fractions were lower, but the relationships between rates measured *in vivo* and in the perfused hemicorpus were similar to those seen in fed rats. 5. The addition of insulin to the perfusate of the hemicorpus prepared from 1-day-starved animals increased the rates of protein synthesis per unit of RNA in gastrocnemius and plantaris muscles to values above those seen in fed animals when measured *in vivo* or in the hemicorpus. Insulin had no effect on the soleus. 6. Overall, the rates of protein synthesis in the hemicorpus differed from those *in vivo*. However, the effect of starvation when measured in the whole animal was very similar to that measured in the isolated rat hemicorpus when insulin was omitted from the perfusate.

The regulation of protein synthesis and breakdown in animal tissues has been studied both in the whole animal, *in vivo*, and in isolated tissues, perfused or incubated *in vitro*. Both approaches have drawbacks. Whereas regulation must ultimately be regarded in the context of the whole animal, with measurements *in vivo* it is often difficult to separate the effects of the particular agent under study from the numerous other regulatory factors which may alter or even reverse the primary change. On the other hand, an isolated tissue, although overcoming this difficulty, has been removed from its normal physical and biochemical environment, and may behave differently from that in the whole animal. For example, activity, innervation and stretch are all believed to play a part in the regulation of muscle protein turnover (see, e.g., Goldberg, 1972; Turner & Garlick, 1974; Gold-

spink, 1977; Palmer *et al.*, 1981). Thus tissues *in vitro* frequently display negative protein balance and may have different rates of protein synthesis from those in the whole animal.

In our studies of the regulation of protein synthesis in skeletal muscle we have adopted the perfused rat hemicorpus preparation, basically as described by Ruderman *et al.* (1971) and Jefferson (1975). This has the advantage over incubated muscle preparations, e.g. diaphragm (Fulks *et al.*, 1975) or soleus or extensor digitorum longus (Li & Goldberg, 1976), that nutrients and hormones reach the muscle via an intact capillary bed. Whereas there have been a few comparisons between approaches *in vivo* and *in vitro* with incubated muscles (Palmer *et al.*, 1981; Goldspink *et al.*, 1983), to our knowledge there have been none with the perfused rat hemicorpus. We have therefore compared rates

of protein synthesis in the perfused hemicorpus with rates in the whole rat measured simultaneously by constant intravenous infusion of [^{14}C]tyrosine. Measurements were made on several different muscles and on the sarcoplasmic and myofibrillar fractions of protein to see whether perfusion altered the pattern of protein synthesis within the hemicorpus.

Materials and methods

Animals

Male Wistar rats, from Charles River, Margate, Kent, U.K., were housed singly in wire-bottomed plastic cages in a humidified temperature-controlled environment with a 12h-light/12h-dark cycle. They were fed *ad lib* on a synthetic powdered diet containing 18% (w/w) casein (Payne & Stewart, 1972) for a minimum of 5 days before infusion or perfusion, when they weighed between 145 and 210g. Food was removed from 'starved' rats 25h before perfusion or infusion. Two rats were perfused each day, starting at about 12:00h and 17:00h. During the period in between, rats from the same group were infused with [^{14}C]tyrosine for measurement of protein synthesis *in vivo*.

Constant infusion of [^{14}C]tyrosine

Rats were immobilized by wrapping in a cloth and infused via a lateral tail vein with L-[U- ^{14}C]tyrosine (2 $\mu\text{Ci/h}$; from Amersham International, Amersham, Bucks., U.K.) for 3h, as described by Garlick *et al.* (1975). At the end of infusion, the cannula was quickly removed, the rat decapitated and blood collected in heparinized tubes. Muscles were rapidly dissected and plunged into liquid N_2 for measurement of the specific radioactivity of free and protein-bound tyrosine, as described below. Rates of protein synthesis were calculated from the specific radioactivities of free and protein-bound tyrosine in the muscles at the end of the infusion as described by Garlick *et al.* (1973). To calculate synthesis rates of sarcoplasmic and myofibrillar proteins, the modification described by Waterlow *et al.* (1978) was applied.

Perfusion of the rat hemicorpus

This was done basically as described by Ruderman *et al.* (1971) and Jefferson (1975). Rats were injected intraperitoneally with sodium pentobarbitone (50mg/kg body wt.) and 5min later with heparin (5000 units/kg body wt.). From the time anaesthesia became apparent, 100% O_2 was passed to the rat through a hood over the head. The operative procedures, including cannulation of the aorta, hemisection above the diaphragm, flushing with 50ml of perfusate and removal of the remaining visceral tissues have been described previously

(Preedy & Garlick, 1981). The perfusate flow rate was adjusted to 10ml/min and the lung was gassed with O_2/CO_2 (19:1) at a flow rate of 300ml/min. The entire apparatus and preparation were kept in a temperature-controlled cabinet maintained at 37.5°C.

The perfusate contained 45ml of washed aged human erythrocytes, 5.25g of bovine serum albumin (Cohn Fraction V), 0.7mg of pyruvate and 100i.u. of heparin. These were made up to 100ml with bicarbonate buffer (Krebs & Henseleit, 1932) and the pH was adjusted to 7.45 with 0.5M-NaOH. An amino acid mixture was also added to give the following concentrations (μM): aspartate, 59; threonine, 550; serine, 269; asparagine, 59; glutamine, 547; glutamate, 124; proline, 328; glycine, 178; alanine, 498; valine, 161; cysteine, 45; methionine, 62; isoleucine, 84; leucine, 154; tyrosine, 63; phenylalanine, 84; lysine, 694; histidine, 72; arginine, 194; tryptophan, 70. In addition, 200mg of glucose and insulin (when appropriate) were added just before perfusion commenced. Insulin was added to a calculated concentration of 25 munits/ml: 5 min after the addition of labelled tyrosine the measured concentration was 24.3 ± 1.8 (4) munits and at the end of perfusion had fallen to 6.1 ± 0.3 (4) munits/ml. All solutions were passed through filters of pore size 0.5 μm before addition, and during perfusion the perfusate flowed through a series of filters with pore sizes decreasing in four steps from 140 to 26 μm .

After a preliminary period of perfusion (15 or 30 min), [^{14}C]tyrosine was added to measure the rate of protein synthesis. This was done by replacing 38ml of the perfusate with a fresh batch containing 4 μCi (28 μmol) of [^{14}C]tyrosine. This 'tyrosine-rich perfusate' had been prepared from the same batch of erythrocytes, and in parallel with the original perfusate, except that during the washing procedure, and in making up the final volume, bicarbonate buffer containing 2.5mM-tyrosine had been used. Then 4 μCi of [^{14}C]tyrosine was added per 38ml of perfusate, which was then left for at least 90 min at 37.5°C before introduction to the perfusion system, to allow equilibration of radioisotope with the intracellular tyrosine pools of the erythrocytes.

Perfusions were then continued for a further period, during which the perfusate was sampled at regular intervals. To terminate perfusions, muscles were rapidly dissected out and plunged into liquid- N_2 . The specific radioactivity of free and protein-bound tyrosine was measured as described below, and rates of protein synthesis were calculated as described in the Results section.

For measurements of the concentrations of adenine nucleotides and phosphocreatine in muscle, hemicorpuses were perfused as described above, except that there was no addition of labelled

tyrosine. Muscles were then clamped in aluminium tongs previously cooled in liquid N_2 and stored at $-196^\circ C$ until analysis. As in other studies, two perfusions were performed each day, and in the period in between similar rats were killed for measurements *in vivo*. These were anaesthetized with pentobarbitone and given O_2 and an injection of heparin as in the preparation of the hemicorpus. When the rat was fully anaesthetized, the muscles were exposed and clamped in pre-cooled aluminium tongs before storage at $-196^\circ C$.

Measurement of tyrosine specific radioactivity and RNA

Muscles from animals infused with [^{14}C]tyrosine or perfused in the presence of [^{14}C]tyrosine were pulverized between pre-cooled aluminium blocks and precipitated in 5 ml of 10% (w/v) trichloroacetic acid. After centrifugation (3000 g, 15 min at $0-4^\circ C$) the supernatant was retained and, after removal of trichloroacetic acid by washing with diethyl ether, was used for measurement of the specific radioactivity of free tyrosine. The precipitate was washed three times with 10–15 ml of ice-cold 2% (w/v) $HClO_4$, resuspended in 0.3 M-NaOH and incubated at $37^\circ C$ for 1 h. Samples were taken for measurement of protein by a modification of the Lowry method (Munro & Fleck, 1969) and the remainder was re-precipitated with 20% (w/v) $HClO_4$. RNA was estimated from the A_{260} and A_{232} of the supernatant (Munro & Fleck, 1969) and the precipitate was washed a further three times with 10–15 ml of 2% $HClO_4$. It was then hydrolysed in 3 ml of 6 M-HCl in the presence of 0.05 ml of 2-mercaptoethanol at $110^\circ C$ for 22 h, for measurement of the specific radioactivity of protein-bound tyrosine. The specific radioactivity of tyrosine in samples derived from rats given constant infusions of [^{14}C]tyrosine was measured by enzymic conversion into tyramine, followed by solvent extraction and a fluorimetric assay (Garlick & Marshall, 1972). Samples derived from perfused hemicorpus were filtered before assessment of radioactivity and assay for tyrosine by the method of Waalkes & Udenfriend (1957). The enzymic method was not necessary for samples for perfusions, since no radioactivity could be detected in any substance other than tyrosine.

Separation of sarcoplasmic and myofibrillar proteins

The method was that of Helander (1961) as modified by Bates & Millward (1983). The separation depends on the solubility of sarcoplasmic proteins in buffers of low salt concentration and myofibrillar proteins in high salt. Only a small fraction is discarded, consisting largely of collagen, which is soluble in neither buffer. Bates & Millward

(1983) have reported that the sarcoplasmic fraction prepared by this procedure is uncontaminated by contractile proteins.

Concentrations of metabolites in muscles

Muscles were ground to a fine powder under liquid N_2 , precipitated in 4 vol. of 0.9 M- $HClO_4$, neutralized to $pH 7.4 \pm 0.1$ with 4 M-KOH and the supernatants stored at $-196^\circ C$. Metabolites were assayed by the following methods: ATP and phosphocreatine (Lamprecht *et al.*, 1974); ADP and AMP (Jaworek & Gruber, 1974).

Other measurements

For measurement of free amino acid concentrations, samples of perfusate plasma (1 ml) or muscle (100 mg, powdered between two aluminium blocks pre-cooled in solid CO_2) were precipitated with 2 ml of ice-cold sulphosalicylic acid after addition of 100 nmol of norleucine as an internal standard. After centrifugation, amino acid concentrations in the supernatant were measured with a Locarte amino acid analyser.

Statistics

Results are presented as means $\pm$ S.E.M., with numbers of observations in parentheses. The significance of difference between groups was assessed by Student's *t* test.

Results

Table 1 shows that perfusion of hemicorpus from fed rats for 60 min, in the presence of a starting concentration of 25 munits of insulin/ml, did not result in significant changes in the concentrations of adenine nucleotides and phosphocreatine compared with values *in vivo*. Measurements were made in both gastrocnemius and quadriceps muscles, and they indicate that the procedures for preparation and perfusion of the hemicorpus leave the pathways for energy generation intact. We have also shown in similar perfusions in the absence of insulin that the concentration of ATP in quadriceps muscle is unaltered by perfusion [6.29 ± 0.30 (9) $\mu mol/g$ in the hemicorpus compared with 5.73 ± 0.18 (6) *in vivo*].

The method of measuring the rate of muscle protein synthesis in the perfused hemicorpus was a modification of that described by Jefferson (1975), in which the labelled amino acid tyrosine replaced phenylalanine. The basis of this technique is that the labelled amino acid is added to the perfusate at a high concentration, so that the specific radioactivity in the tissue rises rapidly to a value similar to that in the perfusate plasma and thereafter remains nearly constant. Fig. 1 shows the specific radioactivity in the hemicorpus at intervals up to 60 min after addition of fresh perfusate containing [^{14}C]tyrosine. During the first 10 min there was a rapid decline in

Table 1. Concentrations of adenine nucleotides and phosphocreatine in muscles *in vivo* and in the perfused hemicorpus. Hemicorpuses from fed rats were perfused for 60 min and muscles were freeze-clamped as described in the Materials and methods section. The perfusate contained 25 munits of insulin/ml. Muscles for measurements *in vivo* were taken by freeze-clamping from rats of the same group as described in the Materials and methods section. Values are expressed in $\mu\text{mol/g}$ wet wt., as means $\pm$ S.E.M., with numbers of observations in parentheses. None of the differences between measurements *in vivo* in the perfused hemicorpus was significant.

Metabolite	Muscle	<i>In vivo</i>	Hemicorpus
ATP	Gastrocnemius	4.72 ± 0.82 (6)	5.69 ± 0.45 (8)
	Quadriceps	5.74 ± 0.51 (6)	5.48 ± 0.40 (8)
ADP	Gastrocnemius	0.76 ± 0.08 (6)	0.83 ± 0.04 (8)
	Quadriceps	0.72 ± 0.09 (5)	0.75 ± 0.06 (8)
AMP	Gastrocnemius	0.074 ± 0.009 (6)	0.091 ± 0.008 (8)
	Quadriceps	0.059 ± 0.008 (4)	0.084 ± 0.011 (8)
Phosphocreatine	Gastrocnemius	10.53 ± 1.66 (6)	13.41 ± 2.98 (6)
	Quadriceps	12.81 ± 1.49 (5)	11.30 ± 1.07 (8)

Fig. 1. Specific radioactivity of free and protein-bound tyrosine in the perfused hemicorpus after addition of labelled tyrosine

After completion of the washout period, 38 ml of recirculating medium was replaced with fresh medium containing $4 \mu\text{Ci}$ ($28 \mu\text{mol}$) of L-[U- ^{14}C]-tyrosine, and groups of three or four preparations were terminated at intervals thereafter. O, Free tyrosine in perfusate plasma; ■, free tyrosine in homogenate of quadriceps; ●, free tyrosine in homogenate of gastrocnemius; Δ, protein-bound tyrosine from gastrocnemius.

the specific radioactivity of free tyrosine in the perfusate plasma, which then continued at a slower rate up to 60 min. In the muscle the free-tyrosine specific radioactivity rose rapidly during the first 10 min, but remained almost constant thereafter. There was no apparent difference between gastro-

cnemius and quadriceps muscles. During the period up to 1 h, the specific radioactivity in protein increased almost linearly with time.

This procedure raised the concentration of tyrosine in the perfusate after 60 min to $248 \pm 34 \text{ nmol/ml}$, compared with $60 \pm 5 \text{ nmol/ml}$ in preparations perfused without additional tyrosine (analysis of samples from four perfusions with high tyrosine and four with normal tyrosine; $P < 0.001$). In quadriceps muscle the corresponding concentrations were 290 ± 18 (4) nmol/g and 94 ± 16 (3) nmol/g respectively ($P < 0.001$). Significant differences in the concentration of no other amino acid were detected in the muscle except for glycine and alanine. The high tyrosine increased the glycine in muscle from 4.28 ± 0.21 to $5.78 \pm 0.42 \mu\text{mol/g}$ ($P < 0.05$) and the alanine from 4.86 ± 0.33 to $6.25 \pm 0.62 \mu\text{mol/g}$ ($P < 0.01$) (analysis of four samples from perfusions with high tyrosine and four with normal tyrosine).

For routine measurement, rates of protein synthesis were calculated from the specific radioactivity of tyrosine in protein (S_B) and that of the free tyrosine in muscle (S_A) 35 min after addition of labelled tyrosine, from the relationship

$$k_s = S_B \times \frac{100}{S_A \times t}$$

where k_s is the fractional rate of synthesis expressed as % of the protein synthesized per day and t is the period of incorporation in days. This assumes that the value of S_A is constant throughout the perfusion, but Fig. 1 shows that this involves only a small error.

Rates of protein synthesis in four muscles from fed rats, measured both *in vivo* and in the perfused hemicorpus, are shown in Table 2. The measurements *in vivo* show that gastrocnemius and plantaris were very similar to each other in terms of their fractional rates of protein synthesis (k_s), the RNA/protein ratio and the rate of synthesis per g of RNA.

The fractional rate of synthesis in quadriceps was also similar to that in gastrocnemius and plantaris, but soleus muscle had a faster rate, which could be explained entirely by a higher RNA/protein ratio, since the synthesis per g of RNA was the same as that in the other muscles. By contrast, when measurements were made in the hemicorpus *in vitro*, there were appreciable differences between muscles in the fractional rates of synthesis. In the gastrocnemius the fractional rate of synthesis was the same as that measured *in vivo*. Since the RNA/protein ratio did not differ between measurements *in vivo* and *in vitro*, the synthesis per g of RNA in the gastrocnemius was also the same. In each of the other three muscles, however, the rates of synthesis measured in the hemicorpus were appreciably faster than those *in vivo*. The discrepancy was greatest for soleus, in which it approached a factor of 2.

Table 3 shows similar comparisons between measurements in the hemicorpus and *in vivo* for rats that had been starved for 1 day. The rates in the

gastrocnemius and plantaris measured *in vivo* were lower in starved than in fed rats. There was a fall in the content of RNA but not of protein, resulting in a fall in the RNA/protein ratio (gastrocnemius, $P < 0.001$; plantaris, $P < 0.05$). There were no changes in synthesis per g of RNA. In soleus starvation had no effect. In the hemicorpus measurements were made in the presence and absence of insulin. Without insulin the values for the gastrocnemius compared very favourably with those measured *in vivo*, as for fed rats. Similarly, in the plantaris and soleus, rates were higher than those measured *in vivo*.

When insulin was added to the perfusate of hemicorpus from starved rats, fractional rates of synthesis were increased in gastrocnemius and plantaris to values similar to those for fed rats, as observed previously by Li *et al.* (1978). There was no increase in soleus. However, the increases in the fractional rates of synthesis were not reversals of the changes seen in starvation, since the RNA/protein

Table 2. Rates of synthesis of mixed muscle proteins from fed rats *in vivo* and in the perfused hemicorpus

Rats were given access to food until measurements began. Rates of protein synthesis *in vivo* were measured by constant intravenous infusion of [^{14}C]tyrosine for 3 h. Rates in the perfused hemicorpus were calculated from the incorporation of label into protein 35 min after replacement of 38 ml of perfusate with an equal volume of fresh medium containing 4 μCi (28 μmol) of [^{14}C]tyrosine. Perfusions were performed in the absence of added insulin. Statistical significance of differences between values *in vivo* and in the hemicorpus: * $P \leq 0.05$, ** $P \leq 0.005$.

Measurement	Muscle	RNA content (mg/g of protein)	Protein synthesis	
			k_s (%/day)	(g of protein/g of RNA per day)
<i>In vivo</i>	Gastrocnemius	7.7 ± 0.3 (7)	10.9 ± 0.4 (7)	14.3 ± 0.8 (7)
	Plantaris	7.0 ± 0.5 (6)	10.3 ± 0.6 (6)	15.1 ± 1.1 (6)
	Quadriceps	—	10.2 ± 0.7 (6)	—
	Soleus	8.8 ± 0.3 (6)	12.8 ± 0.7 (6)	14.6 ± 0.5 (6)
Hemicorpus	Gastrocnemius	7.9 ± 0.5 (6)	10.8 ± 0.4 (6)	13.9 ± 0.9 (6)
	Plantaris	7.2 ± 0.3 (5)	14.6 ± 0.7 (5)**	20.3 ± 0.7 (5)**
	Quadriceps	—	12.2 ± 0.6 (6)*	—
	Soleus	8.8 ± 0.6 (5)	24.2 ± 1.4 (5)**	30.5 ± 4.5 (5)**

Table 3. Rates of synthesis of mixed proteins from 1-day-starved rats *in vivo* and in the perfused hemicorpus

Food was withdrawn from rats 1 day before measurements began. For other details see Table 2. Perfusions were performed either in the absence of insulin or in the presence of 25 munits/ml. Statistical significances between values in the perfused hemicorpus without insulin and *in vivo*, or between values in the perfused hemicorpus with and without insulin: * $P \leq 0.05$, ** $P \leq 0.001$.

Measurement	Muscle	RNA content (mg/g of protein)	Protein synthesis	
			k_s (%/day)	(g of protein/g of RNA per day)
<i>In vivo</i>	Gastrocnemius	5.7 ± 0.3 (5)	7.6 ± 1.0 (5)	13.1 ± 1.1 (5)
	Plantaris	5.0 ± 0.4 (5)	7.8 ± 0.5 (5)	15.7 ± 0.5 (5)
	Soleus	8.1 ± 0.5 (4)	12.5 ± 0.7 (4)	15.6 ± 0.9 (4)
Hemicorpus (no insulin)	Gastrocnemius	6.0 ± 0.2 (5)	7.9 ± 0.7 (5)	13.2 ± 1.0 (5)
	Plantaris	5.3 ± 0.5 (5)	10.3 ± 1.0 (5)*	20.0 ± 3.0 (5)
	Soleus	7.9 ± 0.3 (5)	24.3 ± 1.2 (5)**	31.0 ± 1.9 (5)**
Hemicorpus (plus insulin)	Gastrocnemius	6.3 ± 0.2 (4)	11.0 ± 1.1 (4)*	19.2 ± 1.7 (4)*
	Plantaris	5.2 ± 0.4 (4)	14.9 ± 0.9 (4)*	28.8 ± 0.6 (4)*
	Soleus	8.7 ± 0.2 (4)	22.6 ± 3.9 (4)	30.5 ± 3.9 (4)

ratio did not increase. Instead, there was an elevation of the rate of synthesis per g of RNA relative to the values in hemicorpuscles from both fed rats and starved rats when insulin was not added. This is consistent with the ability of insulin to enhance peptide-chain initiation in muscle (Jeffer-son, 1980).

The relationships between rates in fed and starved rats when measured *in vivo* and in the hemicorpus are shown more clearly by Table 4, which shows the rate in the starved rat as a percentage of that in the fed. This comparison is valid, since the fed and starved rats were drawn from single group of animals. Starvation produced the same proportional change in both the fractional rate of synthesis and the synthesis per g of RNA, whether measured *in vivo* or in the hemicorpus without insulin. When insulin was added to the perfusate of the hemicorpus, however, the apparent changes in synthesis per g of RNA produced by starvation in gastrocnemius and plantaris were quite different from those measured *in vivo*.

With some of the muscles removed in the above experiments, the proteins were separated into two components, the sarcoplasmic and myofibrillar

fractions (Helander, 1961). The sarcoplasmic fraction is that which is soluble in low-salt buffers, and is mainly the cytoplasmic enzymes; the myofibrillar fraction is that which is soluble in high-salt buffers, and is primarily the contractile proteins. There is also a component which is insoluble in both, and this was discarded. Table 5 shows the apparent rates of synthesis of the sarcoplasmic and myofibrillar proteins, both *in vivo* and in the perfused hemicorpus. *In vivo*, in both plantaris and quadriceps muscles from fed rats, the rate of synthesis of the sarcoplasmic proteins was significantly higher than that of the myofibrillar proteins ($P < 0.005$). By contrast, when measured in the hemicorpus the two fractions were no longer significantly different, and in the plantaris the rate of synthesis of the myofibrillar fraction was actually higher than that of the sarcoplasmic proteins. The ratio of rate of synthesis of sarcoplasmic to that of myofibrillar proteins was thus significantly lower when measured in the hemicorpus.

Essentially the same effect was observed in the plantaris from starved rats when the hemicorpus was perfused without insulin. The ratio of sarcoplasmic to myofibrillar protein-synthesis rates was signifi-

Table 4. Rates of synthesis of muscle proteins in 1-day-starved rats expressed as percentages of the rates in fed rats. Rates of synthesis of mixed muscle proteins from fed rats were taken from Table 2 and those for starved rats from Table 3. The fractional rates of synthesis of sarcoplasmic (s) and myofibrillar (m) fractions from plantaris muscle were taken from Table 5. Statistical significances of the differences between rates in fed and starved rats are indicated by the asterisks against the equivalent ratio in the Table: * $P \leq 0.05$, ** $P \leq 0.005$.

Measurement	Gastrocnemius		Soleus		Plantaris		Plantaris fractions	
	k_s	(g of protein/g of RNA per day)	k_s	(g of protein/g of RNA per day)	k_s	(g of protein/g of RNA per day)	s	m
<i>In vivo</i>	70**	92	98	107	76*	104	79*	70**
Hemicorpus (no insulin)	69**	95	100	102	73*	99	74*	66*
Hemicorpus (plus insulin)	102	138*	93	100	99	142**	120	109

Table 5. Rates of synthesis of sarcoplasmic and myofibrillar proteins measured *in vivo* and in the perfused hemicorpus. Separations of sarcoplasmic and myofibrillar proteins were made on portions of muscle from some of the rats described previously, and further details may be found in Tables 2 and 3. Statistical significances of differences between values in the perfused hemicorpus without insulin, and *in vivo*, and between values in the hemicorpus with and without insulin: * $P \leq 0.05$, ** $P \leq 0.005$.

Muscle	Feeding	Measurements	k_s		Ratio (s/m)
			Sarcoplasmic (s)	Myofibrillar (m)	
Quadriceps	Fed	<i>In vivo</i>	11.78 ± 0.82 (5)	9.48 ± 0.53 (5)	1.240 ± 0.034 (5)
		Hemicorpus (no insulin)	12.64 ± 1.32 (4)	12.23 ± 0.96 (5)*	1.055 ± 0.024 (4)**
Plantaris	Fed	<i>In vivo</i>	12.23 ± 0.56 (7)	9.87 ± 0.46 (7)	1.241 ± 0.021 (7)
		Hemicorpus (no insulin)	13.75 ± 0.91 (6)	14.30 ± 1.11 (6)**	0.969 ± 0.032 (6)**
Plantaris	Starved	<i>In vivo</i>	9.72 ± 0.86 (4)	6.86 ± 0.69 (4)	1.442 ± 0.025 (4)
		Hemicorpus (no insulin)	10.13 ± 1.29 (4)	9.41 ± 1.17 (4)	1.075 ± 0.016 (4)**
		Hemicorpus (plus insulin)	16.56 ± 1.31 (3)*	15.57 ± 1.14 (3)*	1.062 ± 0.008 (3)

cantly lower in the hemicorpus than *in vivo*. Addition of insulin to the perfusate stimulated synthesis of both fractions of protein to rates that were not significantly different from those in hemicorpus from fed rats. However, the ratio of sarcoplasmic to myofibrillar protein synthesis was the same as that in hemicorpus from starved rats without insulin, and was different from that in fed rats.

From the point of view of the response to starvation, this caused a significant increase in the ratio of sarcoplasmic to myofibrillar protein synthesis, measured both *in vivo* and in the perfused hemicorpus. Both methods showed that a decrease in synthesis of the two fractions occurred on starvation and that this decrease was slightly greater for the myofibrillar proteins (see Table 4).

Discussion

Despite the frequent use of perfused or incubated preparations for the study of muscle protein metabolism, there have been few comparisons of their performance relative to the situation *in vivo*. Rates of protein synthesis in small muscles incubated *in vitro* have been shown to be lower than those obtained *in vivo* (Palmer *et al.*, 1981; Goldspink *et al.*, 1983), but there appears to be no available information on the perfused rat hemicorpus. This preparation has the apparent advantage over incubated muscles of less damage and disturbance during isolation, and the supply of nutrients and hormones by an intact capillary bed. It might thus be expected to compare more favourably with muscles *in vivo* in terms of rates of protein synthesis. In the present study our measurements *in vivo* and in the hemicorpus were made in rats from a single group, and nearly simultaneously, to ensure that the comparison was fair with regard to food absorption and other possible diurnal variations.

The results shown here indicate that the rates of protein synthesis in the hemicorpus, far from being inhibited, were generally higher than those *in vivo*. Only in the gastrocnemius muscle were the rates obtained by the two methods the same. The discrepancy was greatest for soleus, in which rates in the hemicorpus expressed per g of RNA were 2-fold higher than those *in vivo*. However, when insulin was added to the perfusate of preparations from starved rats, synthesis in both plantaris and gastrocnemius also approached these high rates.

There are several possible explanations for these differences. Firstly, it is important to consider the possibility that the observed discrepancy results from an artifact of measurement, either *in vivo* or in the perfusion system. Such artifacts occur most readily from a failure to define accurately the specific radioactivity of the free amino acid at the site of protein synthesis. With our measurement *in vivo* we used constant infusion of [^{14}C]tyrosine, and it was

assumed that the precursor specific radioactivity was the same as that of the free tyrosine in the tissue. When the label is given as a tracer dose in this way, compartmentation of the free amino acid pool has been shown to give rise to erroneous rates in some tissues, particularly liver and gut (Scornik, 1974; McNurlan *et al.*, 1979). Problems from this source are believed to be minimized when the label is given as a massive (non-tracer) dose, causing 'flooding' of all the free pools of that amino acid to the same specific radioactivity (Scornik, 1974; McNurlan *et al.*, 1979). However, with the massive-dose technique, rates of synthesis in soleus muscle measured *in vivo* are similar to those that we obtained by [^{14}C]tyrosine infusion, and rates as high as those that we observed in soleus of the hemicorpus have not been reported (Garlick *et al.*, 1980; Kelly & Goldspink, 1982). We therefore think it unlikely that the observed discrepancy in synthesis rates results from an error in measurement *in vivo*.

The technique that we used to measure protein synthesis in the perfused hemicorpus was a modification of that of Jefferson (1975), in which labelled tyrosine was substituted for phenylalanine because of the relative simplicity of the assay for tyrosine. The same substitution has been made by Dohm *et al.* (1980) and Goodman *et al.* (1981). The principle underlying the method is that of flooding of the free amino acid pools with a high concentration of labelled tyrosine. The disadvantage of tyrosine is its very low solubility in water, making it difficult to achieve such high concentrations as those obtained by Jefferson (1975) with phenylalanine. In addition, the specific radioactivity of tyrosine in the muscle, averaged over the period of measurement, was significantly lower than that in the plasma (Fig. 1), as was observed by Dohm *et al.* (1980). It is therefore possible, as with the measurements *in vivo*, that the discrepancy in synthesis rates resulted from compartmentation of free amino acid pools. We chose to use the free tyrosine in the muscle for our measurements because, as shown in Fig. 1, the incorporation into protein was linear, suggesting a precursor of constant specific radioactivity. This was more consistent with the curve for free tyrosine in the muscle than that in the perfusate. However, if we had chosen to use the plasma instead, the same differences between apparent rates *in vivo* and in the perfusion, and between different muscles, would have been seen, since the ratio of free-tyrosine specific radioactivity in muscle to that in plasma was the same in all muscles and was similar *in vivo* and in the hemicorpus. Although it has been shown in cultured cells that the aminoacyl-tRNA can sometimes have a specific radioactivity below that of the free amino acid in the tissue, even when flooding has been attempted (Hammer & Rannels, 1981; Hildebran *et al.*, 1981), in perfused tissues this has not

been reported. In liver, heart and lung perfused with high concentrations of amino acids, the specific radioactivities in the tissue, in the plasma and in the aminoacyl-tRNA have been shown to be similar (Khairallah & Mortimore, 1976; McKee *et al.*, 1978; Watkins & Rannels, 1980). Clearly, we cannot rule out compartmentation as the source of the differences between the hemicorpus and the whole rat. However, if it is, the relationship between the specific radioactivities in the tissue, plasma and tRNA must vary considerably between different muscles and between the perfused hemicorpus and the muscle *in vivo*.

Comparison with rates obtained in the perfused hemicorpus by others may be illuminating. The technique of Jefferson (1975) with [^{14}C]phenylalanine has given lower rates of synthesis for fed rats than those in Table 2 (i.e. about 6 g of protein/g of RNA per day in gastrocnemius and 7.5 g of protein/g of RNA per day in soleus: values estimated from the data of Flaim *et al.*, 1980) and the difference between soleus and gastrocnemius was not so pronounced. However, the rate of synthesis that they obtained in tibialis muscle was much lower than in either gastrocnemius or soleus (4.5 g of protein/g of RNA per day), suggesting that quite large variations among muscles do occur. Similarly, Goodman *et al.* (1981) obtained lower rates of synthesis in soleus muscle than those in Table 1, even though their technique, with labelled tyrosine, was very similar to our own. This suggests that our high rates of synthesis in soleus are not an artifact of labelling with this amino acid.

From the separate measurements of synthesis of sarcoplasmic and myofibrillar proteins (Table 5), it appears that most of the discrepancy between measurements in the perfusion and those *in vivo* results from a change in the myofibrillar component, with little apparent change in synthesis of the sarcoplasmic fraction. If precursor compartmentation were responsible for this, it would require that the two fractions of protein were synthesized from different pools of tRNA. Compartmentation of synthesis has been demonstrated for albumin and ferritin in liver (Fern & Garlick, 1976), but whether this resulted from compartmentation of cells synthesizing the two proteins or from intracellular compartmentation (e.g. on free and membrane-bound ribosomes; Ilan & Singer, 1975) is not known. Both these sources of compartmentation are possible in muscle. About 15% of the total protein is thought to be in non-contractile cells (Enesco & Puddy, 1964). This will contribute only to the sarcoplasmic fraction, which is itself only 20–25% of total protein (Young, 1970). Muscle also contains membrane-bound ribosomes. Although the proportion is thought to be small, perhaps as low as 9% (Andrews & Tata, 1971), it has been suggested

that they may be concerned in the synthesis of the sarcoplasmic reticulum (Muscatello *et al.*, 1965), whereas the free ribosomes may be involved in the synthesis of myofibrillar proteins (Lentz, 1969). However, for either of these mechanisms to be responsible for the observed differences in the ratio of sarcoplasmic to myofibrillar protein synthesis, the degree of compartmentation of free amino acid pools would have to be appreciable, even in the presence of high concentrations of tyrosine. By contrast, when compartmentation of synthesis of albumin and ferritin in liver was observed by Fern & Garlick (1976), the experimental model was deliberately chosen to enhance the differences between the various pools. It therefore appears to us unlikely that precursor compartmentation accounts for the apparent differences in the synthesis of sarcoplasmic and myofibrillar fractions in the hemicorpus, compared with that *in vivo*.

Another potential reason for the difference in myofibrillar protein synthesis between that *in vivo* and that in the hemicorpus is the possibility that some newly formed contractile proteins have not yet become fully associated with the myofibrils, as described by Etlinger *et al.* (1975). This would create a larger error with the 35 min incorporation period of the hemicorpus than during 3 h of incorporation of label *in vivo*. However, with the same technique as we used for separating myofibrillar and sarcoplasmic proteins, which is different from that of Etlinger *et al.* (1975), Bates & Millward (1983) were unable to demonstrate any variations in the ratio of myofibrillar to sarcoplasmic synthesis with periods of incorporation between 10 min and 4 h. If the differences in synthesis in the hemicorpus relative to the rates *in vivo* are not artifacts of measurements, there must be some factor in our system of perfusion which is able to stimulate protein synthesis.

Although rates of protein synthesis were different in some muscles of the hemicorpus from those *in vivo*, this did not appear to correlate with a change in the energy status of the muscle. In both quadriceps and gastrocnemius, perfusion did not result in significant changes in measured concentrations of adenine nucleotides or phosphocreatine compared with those *in vivo* (Table 1). Similarly, we do not think that the high concentration of tyrosine in the hemicorpus increased protein synthesis. In preparations with normal concentrations of tyrosine, the concentrations of all other amino acids (except alanine and glycine) were the same as in preparations with high tyrosine (see the Results section). Amino acids such as phenylalanine, which are present at low concentration and are not metabolized by the hemicorpus, should be sensitive indicators of the balance of protein synthesis and degradation. The concentration of phenylalanine in

whole perfusate and in muscle of four hemicorpus perfused with high tyrosine was $66 \pm 2 \mu\text{M}$ and $66 \pm 4 \mu\text{mol/g}$ respectively, compared with $68 \pm 1 \mu\text{M}$ and $62 \pm 6 \mu\text{mol/g}$ in four hemicorpus with normal tyrosine concentrations. This observation of no change in the phenylalanine concentration therefore implies either no change in either synthesis or breakdown or equal and opposite changes in both. The increases in alanine and glycine with the high-tyrosine perfusate (see the Results section) cannot have resulted from metabolism of the high concentration of tyrosine, since 100% of the label could still be isolated as tyrosine after 90 min of perfusion. These results contrast with the hypothesis of Garber *et al.* (1976), who postulated that tyrosine may be a precursor for alanine synthesis in skeletal muscle *in vivo*.

Many other factors in the perfusion medium may introduce potential artifacts. For example, when the albumin was dissolved in buffer to the same concentration as in the perfusate, physiological concentrations of somatotropin (growth hormone), tri-iodothyronine and insulin were observed (Preedy, 1981). Li & Jefferson (1978) have pointed out that purification of bovine serum albumin results in lower rates of protein synthesis in the hemicorpus. Additional impurities may have been introduced by the aged human erythrocytes. The discrepancies between rates *in vivo* and *in vitro* may also be due to other factors, such as the use of anaesthetic and heparin, changes in biochemical characteristics of the perfusate as perfusions progress (e.g. lactate) or even the differences in muscle stretch or immobility of the feet, which are known to alter muscle protein synthesis (Goldspink, 1977; Booth & Seider, 1979).

Perhaps the most important observation is that the effect of starvation, whether observed *in vivo* or in the perfused hemicorpus without insulin, is the same in spite of the discrepancies in the absolute values of the rates obtained. Thus starvation lowered the rate of protein synthesis in gastrocnemius muscle by lowering the ribosome content, but without altering the rate of synthesis per ribosome. This result is rather different from that reported previously, when starvation caused an initial decrease in the rate of synthesis per ribosome, followed by a decrease in ribosome content (see, e.g. Millward *et al.*, 1976; Li & Jefferson, 1978; McNurlan *et al.*, 1982; Garlick *et al.*, 1983). The probable reason for this relates to the time of day when measurements were made. In our previous studies we have made measurements in the morning, when 'fed' animals would usually have full stomachs. In the present study the time needed to prepare the perfusion system necessitated experiments in the afternoon. This is the time when rats feed least, so it is possible that the rates of synthesis obtained were more appropriate to the postabsorptive state. Thus a

decrease in synthesis per ribosome may already have occurred. This is consistent with the observation that insulin, when added to the perfusate of preparations from starved rats, caused an elevation of synthesis above those values seen in the fed rats, which, if post-absorptive, would already have been deficient in insulin. It is also consistent with the finding, in a separate set of perfusions, that insulin also caused a small increase in synthesis (results not shown) in preparations from fed rats.

The apparent sensitivity of the various muscles to starvation was also the same when measured by the two different approaches. Both gastrocnemius and plantaris showed appreciable decreases in synthesis, but soleus appeared to be totally resistant. Similarly, when insulin was added to the perfusate of preparations from starved rats, both gastrocnemius and plantaris responded by increasing synthesis, but soleus was refractory. The relative insensitivity of soleus to some of the factors that regulate protein synthesis in other muscles has been demonstrated previously with perfused or incubated muscles, for example during starvation (Li & Goldberg, 1976; Frayn & Maycock, 1979; Goodman *et al.*, 1981), and in diabetes with both perfused muscle (Flaim *et al.*, 1980) and *in vivo* (Albertse *et al.*, 1980). It has been suggested that this results from the high proportion of slow oxidative fibres in soleus (see, e.g. Goodman *et al.*, 1981). Similarly, systems both *in vivo* and *in vitro* showed that synthesis of the myofibrillar proteins was more sensitive to starvation than was that of sarcoplasmic proteins, as has been shown previously with measurements *in vivo* (Bates & Millward, 1978).

Overall, the present experiments confirm that the perfused muscle is capable of responding to stimuli in much the same way as muscle *in vivo*. A similar relationship between rates measured *in vivo* and during incubation *in vitro* was observed by Goldspink *et al.* (1983) in examining the effects of work overload and denervation on soleus muscle. As in the present study, the effects of treatment were proportionately similar when measured *in vivo*, compared with those seen when the muscle was observed *in vitro*, even though the actual rates obtained differed appreciably. However, what is measured in the perfused preparation may not necessarily be the same as what is occurring in the muscle of the whole animal, and the two approaches have different applications. The perfused muscle is acutely sensitive to substances added to the perfusate [e.g. insulin, Table 3 and Li *et al.* (1978); and amino acids, Li & Jefferson (1978)], as well as to the general metabolic state of the donor animal. Hence, whether or not it reflects events *in vivo* will depend on which of these substances have been added to the perfusate. The value of the perfused preparation, therefore, is not as an alternative to measurement *in*

vivo for assessing rates of metabolism in the whole animal. Rather, it provides a means of elucidating the possible humoral factors responsible for these changes (e.g. insulin), in the relative absence of interference from the other regulatory processes that would occur in the whole animal. For this purpose the rates need not be the same as those *in vivo*, so long as the ability to respond to stimuli is unimpaired.

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