Rates of protein synthesis in skin and bone, and their importance in the assessment of protein degradation in the perfused rat hemicorpus

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The perfused rat hemicorpus preparation, which has frequently been used to study muscle metabolism, contains 39% by weight of non-muscle tissue such as skin and bone. Both the concentration of RNA and the incorporation of [U-¹⁴C]tyrosine into protein indicate that the non-muscle components are more active in protein synthesis than is muscle. These observations have important implications for studies of amino acid metabolism, and in particular for the measurement of muscle protein degradation in the hemicorpus.

Skeletal muscle is the largest single tissue in the mammalian body. To determine the manner in which its protein content is regulated, or its role in amino acid metabolism, a variety of techniques have been developed for studying it *in vitro*. These range from homogenates to preparations such as incubated muscles and perfused systems. Muscles perfused *in vitro* appear to have distinct advantages over incubated or homogenized preparations, such as the ability to deliver a large oxygen supply to the tissue, transfer of material to the muscle via an intact capillary bed, and the availability of a large mass of sample. They have therefore been used to study various aspects of amino acid metabolism (Cross *et al.*, 1974; Ruderman & Berger, 1974; Spydevold, 1976; Caldwell *et al.*, 1978) and of the regulation of protein synthesis and breakdown (Jefferson *et al.*, 1977; Flaim *et al.*, 1978; Dohm *et al.*, 1979; Li *et al.*, 1979).

In many of these studies, particularly those of amino acid uptake and efflux or protein breakdown, various assumptions about the preparation have to be made. The fundamental assumption is usually that it is mainly muscle and that any muscle studied is representative of the entire preparation. We have therefore attempted to assess the validity of this assumption in the isolated perfused rat hemicorpus.

Materials and methods

L-[U-¹⁴C]Tyrosine (10 mCi/mmol) was purchased from The Radiochemical Centre (Amersham, Bucks., U.K.) and an amount containing 250 μ Ci was dissolved in 62.5 ml of 0.9% (w/v) NaCl. Bovine serum albumin (Cohn fraction V) was

obtained from Miles Laboratories (Stoke Poges, Slough, Bucks., U.K.) and a 10% (w/v) solution in Krebs–Henseleit bicarbonate buffer was dialysed against 3×10 vol. of the same buffer for 36 h before use as described by Sketcher (1976). Amino acids were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.) and all other chemicals were from BDH Chemicals (Poole, Dorset, U.K.).

Male Wistar rats were obtained from Charles River (Margate, Kent, U.K.), maintained on a synthetic 18% (w/w) casein diet mixed in our own laboratories and housed in a humidified temperature-controlled room on a 12 h-light/12 h-dark cycle. Animals were allowed free access to food and water up to and including the day on which they were used for perfusion, at a weight of 140–220 g.

The composition of the perfusion medium with the addition of 25 munits of insulin/ml (Preedy *et al.*, 1979), and the apparatus (Sketcher, 1976) have been described. The operative procedure after anaesthesia involved the ligation of the inferior mesenteric, inferior and superficial epigastric, renal, suprarenal, coeliac, superior mesenteric vessels and the pubic-epigastric trunk. The descending colon, bladder, testes, associated reproductive tissue, kidneys, adrenal glands and tail were ligated and removed. The aorta was cannulated at a point just above the diaphragm and the animal transected above the cannulation. Immediately after hemisection, the preparation was flushed with 50 ml of perfusate (10 ml/min), during which the liver, gastro-intestinal tract, fat, abdominal muscle and other unnecessary tissues were removed. The preparation was then transferred to a sealed container and a recirculating system employed. The total volume of perfusate at

this point was 90 ml. In the studies with radioisotope, 4 μ Ci (28 μ mol) of L-[U- 14 C]tyrosine was added to the perfusate 15 min after the end of the preliminary washout period, as described previously (Preedy *et al.*, 1979).

Tissues were prepared and RNA and protein determined as described by McNurlan *et al.* (1979). The specific radioactivity of tyrosine in trichloroacetic acid supernatants and protein hydrolysates was measured by separate assessments of radioactivity (liquid-scintillation spectrometry) and tyrosine (Waalkes & Udenfriend, 1957). This approach was valid, because column-chromatographic analysis of perfusate plasma from a total of 16 perfusions under a variety of conditions showed that $100.5 \pm 0.9\%$ (mean $\pm$ S.E.M.) of the radioactivity was recoverable in tyrosine at the end of 90 min perfusions. Specific-radioactivity measurements of protein from feet, bone and skin were made on fractions soluble in 0.3 M-NaOH after incubation at 37°C for 1 h, and specific radioactivities of protein from muscle were measured on the whole tissue protein. The insoluble component of skin comprised an appreciable proportion of the total protein, but contained no detectable radioactivity and hence did not contribute to the total turnover. Similarly, the contribution of insoluble material in feet, bone and muscle was also negligible.

Results and discussion

Dissection of the hemicorpus preparation after perfusion of dye (Table 1) demonstrates that tissues other than muscle are perfused under the conditions studied. The proportion of non-muscle tissue, 44% by weight, is similar to that reported elsewhere

(Jefferson, 1975), although this value will be dependent upon which of the blood vessels are ligated (see the Materials and methods section) and the treatment of the animal before the perfusion. However, it is exceedingly difficult to occlude the blood supply to all the non-muscle tissues. For example, small branches of the femoral artery, which are difficult to ligate, supply skin, bone, genitalia, subcutaneous fat and mammary glands, as well as muscle (Greene, 1955; Sissons & Grossman, 1953).

Table 1 shows that the skin, feet and bone have a lower protein concentration than the gastrocnemius muscle. It appears therefore that the contribution of the protein of the perfused non-muscle fraction to the overall preparation is small (24%). However, the ratio of RNA to protein in the non-muscle tissues is higher than in muscle, suggesting that these tissues might have a greater ability to synthesize protein.

The results in Table 2 also show that in the perfused hemicorpus all the non-muscle tissues examined incorporate L-[U- 14 C]tyrosine into protein. Rates of protein synthesis can be calculated if it is assumed that the specific radioactivity of free tyrosine in the tissue rises quickly to a constant value after addition of radioisotope. This assumption has been shown to be reasonable for muscle (Preedy *et al.*, 1979; Dohm *et al.*, 1980). In skin and bone a detailed time course has not been measured, but the specific radioactivity at 40 min of perfusion was no different from that at the end. Thus it can be calculated from the data in Table 2 that the fractional rates of protein synthesis are 11.2%/day in gastrocnemius muscle, 17.6%/day in skin, 38.5%/day in feet and 30.7%/day in the tibia. It is also possible to estimate the contribution of the non-muscle tissues to the overall amount of protein

Table 1. *Relative proportions of perfused components and their composition in the isolated rat hemicorpus*

The relative proportions of muscle and non-muscle tissues were determined by the addition of 2 ml of filtered Evans Blue dye to the perfusate at the onset of 40-min perfusions. The proportions of protein and RNA in the muscle and non-muscle tissues were determined on samples removed from a separate set of perfusions. The entire skin covering the preparation was removed for analysis and the feet were severed from the ankle joint. The gastrocnemius and tibia were taken to represent the musculature and bone mass respectively. Results are means $\pm$ S.E.M. for between four and six observations. Significance of difference between muscle and non-muscle tissues was assessed by Student's *t* test: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

	Percentage of total weight	Percentage of total protein	Protein concn.† (mg/g wet wt.)	RNA concn. (mg/g wet wt.)	RNA/protein (mg/g)
Perfused tissues					
Muscle	56.3 $\pm$ 1.3	68.4	156 $\pm$ 4	1.47 $\pm$ 0.11	9.4 $\pm$ 0.8
Skin	20.4 $\pm$ 0.8	10.0	63 $\pm$ 6***	1.11 $\pm$ 0.10	18.3 $\pm$ 2.1**
Bone	9.6 $\pm$ 0.2	6.9	92 $\pm$ 2***	2.44 $\pm$ 0.15**	26.7 $\pm$ 2.1***
Feet	4.0 $\pm$ 0.1	3.1	99 $\pm$ 3***	1.15 $\pm$ 0.16	13.5 $\pm$ 0.8*
Other	3.4 $\pm$ 0.2	4.0‡			
Unperfused tissue	6.5 $\pm$ 0.2	7.6‡			

† Protein soluble in 0.3 M-NaOH.

‡ Assumed to have a protein concentration of 150 mg/g.

Table 2. Concentration and specific radioactivities of L-[U-¹⁴C]tyrosine in muscle and non-muscle tissues of the isolated perfused hemicorpus

Rats were prepared for perfusion as described in the text: 20 min after the aorta was cannulated, L-[U-¹⁴C]tyrosine was added to the perfusate. The entire skin covering the hemicorpus, and feet from the ankle joint, were analysed. Gastrocnemius and tibia were taken to represent muscle and bone respectively. The concentrations and specific radioactivities of free tyrosine in the tissue and the specific radioactivities of tyrosine in protein are those values occurring 75 min after the addition of [U-¹⁴C]tyrosine to the perfusate. At this point the specific radioactivity of free tyrosine in plasma ranged from 169 to 189 d.p.m./nmol. Results are presented as means $\pm$ S.E.M. for four to six observations. Significance of difference between muscle and non-muscle tissue was determined by Student's *t* test; ***P* < 0.01; ****P* < 0.001.

	Free tyrosine concentration (nmol/g wet wt.)	Specific radioactivity (d.p.m./nmol)		
		Free	Protein	10 ³ $\times$ protein/free
Muscle	344 $\pm$ 25	143 $\pm$ 4	0.83 $\pm$ 0.07***	5.85
Skin	616 $\pm$ 70**	32 $\pm$ 4***	0.29 $\pm$ 0.05***	9.14
Bone	—	57 $\pm$ 5***	0.91 $\pm$ 0.12	15.99
Feet	393 $\pm$ 9	90 $\pm$ 2***	1.80 $\pm$ 0.07***	20.50

synthesized in the hemicorpus. The number of milligrams of protein synthesized in a particular organ is obtained by multiplying the fractional synthesis rate by the protein content (from Table 1). In a preparation from a 200g rat, therefore, non-muscle tissues synthesize 19mg of protein/h, compared with 28mg/h in the muscle. In this calculation we have assumed that the gastrocnemius muscle is representative of the entire musculature and that the skeletal mass is represented by the tibia.

A potential source of error with these calculations is that we do not know the specific radioactivity of the free amino acid at the site of protein synthesis. Without making measurements on aminoacyl-tRNA, it is difficult to obtain the true synthesis rates. Although we have used the specific radioactivity of free tyrosine from the tissue in the calculations above, it is possible that it may be just as suitable to use that in the plasma (Waterlow *et al.*, 1978). Therefore, if we recalculate these rates using the specific radioactivity of free tyrosine in plasma, we arrive at a lower estimate of the proportion of total synthesis in the non-muscle fraction. This is because the ratio of intracellular to plasma specific radioactivity is lower in non-muscle tissues than in muscle (see Table 2), possibly because of accelerated proteolysis during perfusion (which might also explain the high concentration of free tyrosine in skin). Thus, in this calculation 6 mg of protein/h would be synthesized by the non-muscle tissues and 23 mg/h by the muscle.

At the end of a separate set of perfusions, the skin covering the preparation was discarded and the gastrocnemius muscle and remaining carcass were homogenized separately. Total synthesis in muscle and remaining carcass (minus skin) was determined from measurements of the specific radioactivity of

tyrosine in protein and of free tyrosine in plasma. The assumption was also made that the gastrocnemius was representative of total muscle and that the composition of the muscle and remaining carcass was as shown in Table 1. In this instance the synthesis of 11 mg of protein/h was attributable to the non-muscle tissues, compared with 18 mg/h in the muscle (similar to previous results). The higher estimate of synthesis in non-muscle tissues may have resulted from a very rapid synthesis in components that were not listed in Table 2.

It is clear, therefore, that however we calculate the rates of synthesis, the non-muscle components contribute a large percentage of total protein synthesis in the hemicorpus. This will influence perfusion studies in two ways. Firstly, measurements which assume that changes in the concentration of a free amino acid in the perfusate result solely from changes in muscle metabolism must be interpreted with caution. For example, in studies of amino acid metabolism in perfused tissue (e.g. Ruderman & Berger, 1974; Spydevold, 1976; Caldwell *et al.*, 1978) it is possible that an appreciable proportion of net uptake or efflux can be attributed to the non-muscle components, if their relative rates of protein synthesis can be taken as indicative of their contribution to net amino acid flux. The same argument may apply to measurements of amino acid uptake or output *in vivo* from the arterio-venous difference in concentration across the leg (e.g. Ballard *et al.*, 1976; Wahren *et al.*, 1976; Schrock *et al.*, 1980).

The second way in which our results will influence studies made in perfused preparations relates to the estimation of rates of protein breakdown in muscle (e.g. Dohm *et al.*, 1979; Li *et al.*, 1979). With the method described by Jefferson *et al.* (1977), the rate of protein degradation of the total hemicorpus

protein is obtained, and it is possible that this may be different from the rate occurring in muscle itself. In addition, this technique of measuring protein breakdown (Jefferson *et al.*, 1977) requires estimates of the amount of radioisotope and free amino acid in the total preparation, which appear to have been determined by assuming that a single muscle is representative of the whole hemicorpus. The data in Table 2 show that, for [^{14}C]tyrosine under our conditions of perfusion, this assumption is not true.

Our observations on the non-muscle tissues of the hemicorpus do not necessarily imply that the results of perfusion studies *in vitro* are invalid. The isolated hemicorpus preparation, for instance, is approx. 35% of the total body weight of fed rats. Therefore, meaningful results concerning 'peripheral' metabolism can be implied. However, existing data from perfused 'muscle' preparations should be reinterpreted in the light of the probable contribution of non-muscle tissues to overall amino acid or protein metabolism.

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