Measurement of the synthesis rates of collagens and total protein in rabbit muscle

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1. Collagen- and total-protein-synthesis rates were determined in rabbit muscle by continuous infusion of radioactive proline. 2. The precursor pool of free proline used for collagen synthesis was defined by measuring the specific radioactivity of hydroxyproline in isolated type I procollagen. The specific radioactivities of type I procollagen were about 40% of those for free proline in the homogenate. 3. The mean ratio ($\pm$ S.E.M.) between the fractional synthesis rates of muscle collagen and total protein was 0.99 ± 0.10 , where the total protein values were based on specific radioactivities of the homogenate free proline pools. 4. Types I, III and V collagen were solubilized by pepsin and isolated by fractional precipitation with NaCl. The fractional synthesis rates of types I and III collagens were very similar. Type V collagen samples had higher specific radioactivities than the other collagens, but this was not necessarily indicative of a higher rate of synthesis because of uncertainty about the cellular origin of this collagen and, hence, the specific radioactivity of its precursor proline pool.

As the major protein of connective tissues, collagen plays a major role in the structural characteristics of muscle. Three different isomorphous forms of collagen (see Miller, 1976) were shown to be present in skeletal muscle, having distinct anatomical distributions (Duance *et al.*, 1977) consistent with the different properties of these various collagen types. Thus relatively large type I collagen fibres with a high tensile strength form the epimysial sheath, whereas fine fibres of type III collagen giving rise to a high structural compliance are the major components of both the perimysium that encloses muscle bundles and the endomysium surrounding individual muscle fibres. Type V collagen (Burgeson *et al.*, 1976; Duance *et al.*, 1977; Bentz *et al.*, 1978; Rhodes & Miller, 1978) is present in the endomysium and appears to form part of an amorphous basement-membrane structure.

A knowledge of the relative rates of turnover of the different collagen types and of the myofibrillar and sarcoplasmic proteins is fundamental to an understanding of the mechanism and control of muscle growth. The myofibrillar and sarcoplasmic proteins have similar turnover rates in both chicken and rabbit muscle (Laurent *et al.*, 1978a; Loble & Lovie, 1979), but, in one of the few studies of muscle collagen synthesis *in vivo*, Laurent *et al.* (1978b) found that the fractional synthesis rate of intra-

muscular collagen in chicken was much lower than that of total muscle protein.

In the present paper, the measured synthesis rates of total muscle protein are compared with those of the various collagen types isolated from muscle after treatment with pepsin. The specific radioactivity of the free pool of proline used for collagen synthesis was determined by isolating collagen precursor proteins that were shown to have reached plateau values during the times of infusion (Robins, 1979). Protein-synthesis rates were calculated according to the kinetic analysis described by Loble *et al.* (1980).

Experimental

Materials

L-[5-³H]Proline (sp. radioactivity 12 Ci/mmol) and L-[U-¹⁴C]proline (sp. radioactivity 300 Ci/mol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Scintillation fluid (NE 260) was obtained from Nuclear Enterprises, Edinburgh, Scotland, U.K., and pepsin (twice crystallized, freeze-dried; ex pig stomach mucosa) from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. All other chemicals were of analytical grade.

Rabbit infusions

Continuous infusions of radioactive amino acids were performed in New Zealand White rabbits weighing 800–1400 g (Nicholas *et al.*, 1977). Infusions were continued for up to 10 h, during which time the animals were permitted free movement and access to food and water. Experiments where both [^3H]proline and [^{14}C]proline were infused were performed as described by Lobley *et al.* (1980).

Extraction of collagen precursors

Readily dissectable muscles of the back and thigh, representing about 10% of the body weight of the animals, were rapidly cleaned of adhering fascia and tendon attachments and minced in a Moulinex type RLIA mincer. After removal of a small portion for the measurement of specific radioactivity in total protein, the tissue was suspended in 2 vol. of extraction buffer, which contained 150 mM-NaCl, 50 mM-Tris/HCl buffer, pH 7.5, 25 mM-EDTA, 10 mM-N-ethylmaleimide, 10 mM-iodoacetate, 0.2 mM-2-aminopropionitrile and 0.2 mM-phenyl-methanesulphonyl fluoride. The suspension was homogenized for 20 s at full speed with a Polytron PT 10/35 homogenizer and was then centrifuged at 27000 g for 15 min. In one experiment, a second extraction of the residue was done under similar conditions and the supernatants were combined. Types I and III collagen precursors in the supernatant solutions were isolated by $(\text{NH}_4)_2\text{SO}_4$ precipitation and DEAE-cellulose chromatography as described by Robins (1979). All of the extraction and purification procedures were performed at 4°C.

Extraction of collagens

In preliminary experiments, attempts were made to remove the bulk of the myofibrillar proteins by extraction with 1.1 M-KI/0.1 M- K_2HPO_4 (Helander, 1957), but this resulted in removal of a significant proportion of the radioactive collagen. The residue from the initial extraction was therefore submitted to treatment with pepsin directly to solubilize all of the intramuscular collagen. The tissue was suspended in 0.5 M-acetic acid (10 ml/g wet wt.), stirred overnight to allow swelling, and then treated with pepsin (20 mg/g of muscle). The suspension was stirred for a further 48 h, after which time the insoluble residue was removed by centrifugation and the pepsin treatment was repeated. The combined supernatants were adjusted to pH 7.4 with 3 M-NaOH, solid NaCl was added to a final concentration of 3.5 M and, after stirring of the mixture for 18 h, the precipitated collagen was centrifuged down at 27000 g. The precipitate was re-dissolved in 0.5 M-acetic acid and was dialysed against four changes (each 2 litres) of 1 M-NaCl/20 mM-Tris/HCl buffer, pH 7.6: some undissolved material, which contained less than 1% of the collagen, was removed by centrifugation and

discarded. Types I and III collagen were separated by fractional precipitation at 1.6 M-, 1.8 M-, 2.0 M-, 2.4 M- and 2.8 M-NaCl: type V collagen was precipitated by dialysis of the final supernatant solution against four changes (2 litres) of 20 mM- Na_2HPO_4 , pH 9. The collagen samples and pepsin-treated residues were dialysed against water and freeze-dried. All procedures were performed at 4°C.

Analytical techniques

The extraction of amino acid free pools from muscle and the preparation of protein samples were performed as described previously (Nicholas *et al.*, 1977; Robins, 1979). Protein samples were hydrolysed in 5.7 M-HCl at 105°C for 24 h, and the acid was removed by evaporation *in vacuo*. Samples were fractionated by using a Locarte analyser, and portions of individual amino acids in the relevant pooled fractions were re-chromatographed to determine amino acid content and analysed for radioactivity. In the free pool samples from muscle, an unidentified ninhydrin-positive component that co-chromatographed with proline in the initial fractionation was present but was separated by slight alteration of the chromatographic conditions (Reeds *et al.*, 1980): this contaminant was shown to be non-radioactive.

Hydroxyproline analyses for the determination of collagen contents were done by the method of Firschein & Shill (1966) but with 2-methoxyethanol instead of propan-2-ol. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Robins (1979). Radioactivity determinations with NE 260 scintillation fluid were made in a Beckman LS-345 counter.

Calculation of fractional synthesis rates

Fractional synthesis rates (FSR), expressed as a percentage of the total protein pool synthesized per day, were calculated from:

$$\text{FSR} = \frac{S_B}{\int_0^t S \cdot dt} \times 100$$

where S_B is the specific radioactivity of protein-bound amino acid after an infusion time, t (days), and $\int_0^t S \cdot dt$ defines the specific radioactivity of the precursor for protein synthesis. $\int_0^t S \cdot dt$ is given by $(S_{\text{max.}}/\phi)(\phi t + e^{-\phi t} - 1)$, where $S_{\text{max.}}$ is the specific radioactivity at plateau and ϕ is a first-order rate constant (Lobley *et al.*, 1980). For calculation of collagen-synthesis rates $S_{\text{max.}}$ was taken as the specific radioactivity of hydroxyproline in type I procollagen, whereas for total protein synthesis calculations were based on specific radioactivities of the muscle-homogenate free proline for $S_{\text{max.}}$. The rate constants used were those for blood that were either calculated for individual animals from the

results of double-isotope infusions or were taken as mean values (see the legend to Table 2).

Results

Rabbit infusions

The radioactivity measurements of free proline in the blood and muscle homogenate (Table 1) showed that plateau conditions were attained in the blood within 2–3 h, as evidenced by the similarities in the ³H/¹⁴C ratios between the blood and infusate. By contrast, the ³H/¹⁴C ratio in the muscle homogenate was considerably higher than in the infusate, indicating a lower rate of equilibration (see Lobley *et al.*, 1980). The mean ratio (±s.d.) between the specific radioactivities for free proline in the homogenate and blood was 0.58 ± 0.17.

The specific radioactivities of the isolated collagen precursors were always lower than those of the free pool (Table 1), and, although there was no fixed relationship, most of the values for type I collagen precursors were about 40% of the corresponding homogenate specific radioactivities. The ³H/¹⁴C ratios for the type I precursor fraction confirmed that the turnover of this protein in muscle was rapid and that the specific radioactivities attained after infusion times of longer than 3 h were close to their plateau values. For two of the three double-label experiments, the ³H/¹⁴C ratio for type I collagen precursors was lower than that of free proline in the homogenate (Table 1), indicating that the rate of equilibration of the proline pool used for collagen

synthesis in muscle was faster than that of the total muscle-homogenate free pool. For this reason, rates of collagen synthesis were based on equilibration rate constants of the blood (ϕ) rather than of the muscle homogenate (ν). The specific radioactivities of the type III collagen precursors were generally lower than their type I counterparts, and this, together with the higher ³H/¹⁴C ratios (Table 1), indicated a slower apparent turnover rate. In common with results for skin (Robins, 1979), electrophoretic analysis of this fraction revealed a high proportion of intermediates of procollagen processing that have a lower specific radioactivity (S. P. Robins, unpublished work). Where the extraction procedure was repeated (infusion 4), the larger proportion of intermediates that was present probably accounted for the greater difference in specific radioactivities between the type I and type III collagen precursor fractions (Table 1). In view of these considerations, the specific radioactivities of the type I collagen precursors were assumed to represent the proline pool specific radioactivity for calculating the synthesis rates of both types I and III collagen.

Collagen synthesis

The yields of collagen precursors isolated by DEAE-cellulose chromatography were approx. 200 µg/100 g of fresh muscle for type I and about twice that amount for type III collagen precursors. The predominance of the latter is related to differences in the rates of procollagen processing and

Table 1. Continuous infusions of radioactively labelled proline into rabbits

After infusions for the times indicated, specific radioactivities of free proline in deproteinized extracts of whole blood and muscle homogenate and of hydroxyproline in the isolated collagen precursors were determined. For dual-label experiments, the animals were receiving both [³H]proline and [¹⁴C]proline at the end of the experiment. For further details see the Experimental section.

Infusion no.	Infusion time (h)	Proline label	Specific radioactivity (d.p.m./nmol)			
			Whole blood (S_p)	Muscle free pool (S_h)	Type-I-collagen precursors (S_I)	Type-III-collagen precursors (S_{III})
1	10	^3H	543	409	179	150
2	{ 6	^3H	448	363	129	—
		^{14}C	103	68	21	—
3	5	^3H	957	374	220	230
4	6	^3H	989	411	142	80
5	{ 8	^3H	1665	884	323	232
		^{14}C	460	140	54	17
6	{ 6	^3H	772	460	319	248
		^{14}C	265	98	91	52
$^3\text{H}/^{14}\text{C}$ ratio						
		Infusate	S_p	S_h	S_I	S_{III}
2		4.19	4.35	5.34	6.14	—
5		3.05	3.62	6.31	5.98	13.65
6		2.65	2.91	4.69	3.51	4.77

does not reflect the proportions of the two collagen types in muscle (see below). The extracted procollagens contained less than 0.1% of the total hydroxyproline in muscle and less than 5% of the hydroxy[^3H]proline. This fraction was therefore neglected in calculating collagen-synthesis rates.

Pepsin treatment of the extracted muscle samples resulted in solubilization of over 95% of the collagen. The fractions obtained by salt precipitation were characterized by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Material that was precipitated at 1.6M- and 1.8M-NaCl, representing 15–20% of the total, was predominantly type III collagen; in the subsequent precipitates up to 2.8M-NaCl, only type I collagen was detected. The precipitate obtained by dialysis against 20mM- Na_2HPO_4 , which corresponded to less than 1% of the total collagen precipitated, contained mainly type V collagen, but with some contamination by type I collagen and type I trimer (Jimenez *et al.*, 1977).

By summation of the various fractions obtained, the mean ($\pm$ S.D.) collagen content of the muscle samples was $2.5 \pm 0.05\%$ of the total muscle protein.

In Table 2, the calculated fractional synthesis rates of collagen are compared with those of muscle total protein. The latter were based on incorporation rates of [^3H]proline, where the precursor-pool

specific radioactivity was assumed to be that of the homogenate free pool. The mean ratio ($\pm$ S.E.M.) between the fractional synthesis rates of collagen and total muscle protein was 0.99 ± 0.10 ; collagen synthesis therefore constituted about 2.5% of total protein synthesis in muscle. The synthesis rates of type I and type III collagen were very similar in all of the tissues examined. The specific radioactivities of type V collagen were, however, up to twice those of the corresponding type I collagens, and, because of the contamination of the preparations of type V collagen, this difference is probably an underestimate. As discussed below, no fractional synthesis rates have been tabulated for type V collagen owing to the uncertainty with regard to its precursor pool.

Discussion

In the present study, the specific radioactivity of the precursor pool of proline used for collagen synthesis was defined by measuring the specific radioactivity in type I procollagen, which was shown by double-isotope experiments to have reached a plateau value, certainly within the longer times of infusion (^3H label). In common with the results for skin (Robins, 1979), the precursor-pool specific radioactivity was much lower than that of the tissue homogenate, indicating that, in the collagen-producing cells at least, there is a relatively large contribution of unlabelled proline to this pool from protein degradation and synthesis *de novo*. In addition, these results suggest that the common assumption that collagen and non-collagenous protein in muscle are synthesized from the same precursor pool may not be valid. The occurrence of intracellular degradation of procollagen, as has been demonstrated in cultured fibroblasts (Steinberg, 1973, 1978) and other systems (Bienkowski *et al.*, 1978; Holmes & Trelstad, 1979), would not seriously affect the labelling kinetics of procollagen, although, if this were the case, the collagen-synthesis rates determined would be less than their absolute values.

From the measured precursor specific radioactivity, the calculated fractional synthesis rate of muscle collagen was similar to that of the total mixed protein, where the latter is based on the rate constant for blood (Lobley *et al.*, 1980) and the precursor specific radioactivity of the homogenate free proline. Whether the latter assumption is valid is not known, but, if the specific radioactivity of extracellular (blood) free proline were used, then the value for total muscle protein synthesis would be considerably less than that for collagen. These relatively rapid rates of collagen synthesis compared with muscle total protein in growing rabbits are in contrast with the results obtained by Laurent *et al.* (1978b) for adult chickens. In the latter

Table 2. *Specific radioactivities and fractional synthesis rates of muscle collagens*

From the specific radioactivities of the isolated collagens, the fractional synthesis rates were calculated by using the specific radioactivities of type I procollagen (Table 1) to define the precursor amino acid pool. For infusions 1 and 2, the collagen values represent a mean for the total muscle collagen, as no fractionations were carried out. Muscle mixed-protein-synthesis rates are based on plateau radioactivities in the homogenate and rate constants for blood (ϕ) that were calculated individually from isotope ratios (Lobley *et al.*, 1980) for infusions 2 (26 days $^{-1}$), 5 (22 days $^{-1}$) and 6 (19 days $^{-1}$); for infusions 1, 3 and 4, a mean value of $\phi = 32$ days $^{-1}$ was used.

Infusion no.	Hydroxyproline specific radioactivity in collagen	Fractional synthesis rate (%/day)				
		Collagen	Muscle protein			
1	5.70	8.3	6.5			
2	2.53	9.2	9.1			
	Collagen type			Type		
	I	III	V	I	III	
3	1.08	0.95	1.84	2.8	2.3	4.5
4	0.76	0.64	1.19	2.4	2.1	2.7
5	3.72	3.55	5.03	4.0	3.8	3.9
6	3.91	4.20	5.92	6.2	6.7	5.4

experiments, collagen-synthesis rates in skeletal muscle of 0.59%/day were recorded, compared with 17%/day for the total mixed protein; during rapid growth induced by stretch, the collagen-synthesis rates increased to 4.5%/day, a value similar to the present results for growing rabbits, but at this stage total muscle protein exhibited a 7-fold higher rate of synthesis (Laurent *et al.*, 1978b). Although the collagen-synthesis rates determined by Laurent *et al.* (1978b) were based on specific radioactivities of the homogenate free proline pools rather than on values for procollagen as in the present study, it is unlikely that this could account entirely for the apparent differences in the relative synthesis rates of collagen and total mixed protein that occur in chicken muscle.

The distribution of the various collagen types in different proportions within the muscle (Duance *et al.*, 1977) introduces an additional problem in determining collagen-synthesis rates. Some doubt exists as to the cellular origin of muscle collagens, but it is generally accepted that the fibroblasts are responsible for most of the type I and type III collagen synthesis. No independent assessment of the precursor-pool specific radioactivity for type III procollagen synthesis could be obtained, as the presence of intermediates that co-chromatographed with type III procollagen on DEAE-cellulose resulted in the isolation of a fraction that had not reached equilibrium. The plateau specific radioactivities were assumed to be the same as the corresponding values determined for type I procollagen. This is a reasonable assumption, as fibroblasts have been shown to be capable of synthesizing both type I and type III collagens simultaneously (Gay *et al.*, 1976). Observations *in vitro* have shown that fibroblasts synthesize these two collagen types in a consistent proportion (Hance & Crystal, 1977), but that the ratio may be altered by various environmental effects, such as oxygen concentration (Bhatnagar *et al.*, 1978) or serum factors (Narayanan & Page, 1977). Presumably, therefore, the presence of a greater proportion of type I collagen in the epimysium and the predominance of type III collagen in the perimysium reflect differences in the relative proportions of these collagens synthesized by the distinct populations of cells that are present within these areas of the muscle (Enesco & Puddy, 1964). The assignment of similar fractional synthesis rates to type I and type III collagen is dependent on the assumption that fibroblasts throughout the muscle are utilizing proline pools of the same specific radioactivity.

There is no information about the precursor-pool specific radioactivity for measurement of type V collagen synthesis, as a precursor protein could not be isolated: indeed, some basement-membrane forms of procollagen appear not to undergo extracellular

processing (Minor *et al.*, 1976). Clearly, if the precursor pool is the same as that used for type I procollagen synthesis, this would indicate a fractional synthesis rate of type V collagen twice that for type I collagen (Table 2). The higher specific radioactivities of type V collagen may, however, be related to differences in cellular origin of the collagens. By using immunological techniques, Bailey *et al.* (1979) showed that myoblasts synthesize type V collagen during differentiation, and production of collagen of unspecified type by myotubes has been demonstrated (Schubert *et al.*, 1973; Lipton, 1977; Garrels, 1979). If type V collagen is, in fact, synthesized by cells other than fibroblasts than the observed differences in specific radioactivity may be due solely to differences in the precursor pools, especially as the specific radioactivity of the free proline pool for type I collagen synthesis was less than half that in the muscle homogenate (Table 1).

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