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Characterization of muscle epimysium, perimysium and endomysium collagens

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In the past it has been proven difficult to separate and characterize collagen from muscle because of its relative paucity in this tissue. The present report presents a comprehensive methodology, combining methods previously described by McCollester [(1962) *Biochim. Biophys. Acta* **57**, 427–437] and Laurent, Cockerill, McAnulty & Hastings [(1981) *Anal. Biochem.* **113**, 301–312], in which the three major tracts of muscle connective tissue, the epimysium, perimysium and endomysium, may be prepared and separated from the bulk of muscle protein. Connective tissue thus prepared may be washed with salt and treated with pepsin to liberate soluble native collagen, or can be washed with sodium dodecyl sulphate to produce a very clean insoluble collagenous product. This latter type of preparation may be used for quantification of the ratio of the major genetic forms of collagen or for measurement of reducible cross-link content to give reproducible results. It was shown that both the epimysium and perimysium contain type I collagen as the major component and type III collagen as a minor component; perimysium also contained traces of type V collagen. The endomysium, the sheaths of individual muscle fibres, was shown to contain both type I and type III collagen as major components. Type V collagen was also present in small amounts, and type IV collagen, the collagenous component of basement membranes, was purified from endomysial preparations. This is the first biochemical demonstration of the presence of type IV collagen in muscle endomysium. The preparation was shown to be very similar to other type IV collagens from other basement membranes on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and was indistinguishable from EHS sarcoma collagen and placenta type IV collagen in the electron microscope after rotary shadowing.

Collagen has long been known to form the main structural constituent of the connective tissue of muscle, providing a network of fibres throughout the body of each muscle. In skeletal muscle the tendon is continuous with both the epimysium (the muscle sheath) and the proliferative perimysium, which forms the bulk of the network mentioned above. At the microscopic level each muscle fibre is surrounded by a basement-membrane sheath, the endomysium, which has an associated reticular layer of fine collagen fibres (Hamm, 1965). The collagen network is continuous throughout the muscle from the endomysium to the tendon. In this way the force of muscle contraction is effectively and efficiently transmitted through the connective tissue to the bone.

Collagen forms only 1–9% of the fat-free dry mass of muscle (Bendall, 1967; Lawrie, 1979), but has been shown to be present in at least four different genetic forms in this tissue. The immunolocalization studies carried out by Duance and his co-workers on normal bovine and human muscle (Bailey *et al.*, 1979*a*,*b*; Duance *et al.*, 1980*a*,*b*) showed that the perimysium, those tracts of connective tissue surrounding bundles of muscle fibres, contained types I and III collagen, whereas the endomysium contained types I, III, IV and V collagen. An early attempt at the biochemical identification of different collagen types in muscle showed the presence of type I and III collagen in crude perimysial preparations, and type V was purified with types I and III from separated myofibres (Bailey & Sims, 1977).

Recently, much attention has been focused on the role of connective tissue proliferation in diseases such as muscular dystrophy, polymyositis and muscle fibrosis associated with systemic

Abbreviation used: SDS, sodium dodecyl sulphate.

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sclerosis. In general, most studies have been by immunomicroscopy (Duance *et al.*, 1980*a*,*b*; Stephens *et al.*, 1982; Black *et al.*, 1983) or by cell or tissue culture from affected biopsy samples (Hauschka & Konigsberg, 1966; Elsdale & Bard, 1972; Lipton, 1977; Stephens *et al.*, 1980) or at the ribosomal level (Ionasecu *et al.*, 1980). Direct biochemical analysis of the types and amounts of collagen in discrete muscle connective-tissue compartments has proven impossible, owing to the lack of a convenient method for the bulk separation of epimysium, perimysium and endomysium.

McClain (1969) devised a procedure for the preparation of muscle connective tissue by grinding frozen samples at two different speeds at −20°C and then filtering the blended samples through a stack of sieves. Unfortunately, the author admitted that the final preparation, though free of mucle protein, contained both perimysium and endomysium in unknown proportions. More recently, Fujii & Murota (1982) described a simple method for the preparation of washed muscle connective tissue, but again this procedure yielded a mixture of both perimysium and endomysium. As lesions in these two quite different connective tissues have been shown to occur in muscular dystrophy (Duance *et al.*, 1980*a*), and the pathology of muscle basement-membrane is of considerable interest in a variety of disorders, a method is needed that can preparatively differentiate between the perimysium and endomysium.

We now report a procedure that combines an original method of McCollester (1962) for the preparation of endomysial 'ghosts', extraction solvents of Hasselbach & Schneider (1951) and Laurent *et al.* (1981) and new procedures originated in this laboratory. The method allows the selective isolation and purification of epimysium, perimysium and endomysium and therewith the collagens from each. Finally, we report the first preparation and partial characterization of muscle endomysial basement-membrane type IV collagen.

Materials and methods

Materials

Bovine skeletal muscle was obtained fresh at slaughter and was treated immediately. All chemicals were of the highest analytical grade, and all organic solvents were high-pressure-liquid-chromatography grade. 7-Chloro-4-nitrobenz-1-oxa-1,3-diazole was obtained from Sigma Chemical Co., Poole, Dorset, U.K., KB^3H_4 was from Amersham International, Amersham, Bucks., U.K.

Solubility of aldimine-cross-linked collagen in SDS

The stability of aldimine (heat- and acid-labile)-cross-linked collagen to SDS was tested to optimize conditions for washing muscle connective tissue. It was necessary to ensure that this reagent did not lead to undue solubilization of cross-linked collagen, so that assays on insoluble material would be representative of all the cross-linked collagen. Rat tail tendon, which contains only the aldimine hydroxylysinonorleucine, was chosen as a model tissue. Six batches of approx. 500mg wet wt. of rat tail tendon were immersed in 25ml of 1% (w/v) SDS at room temperature and were stirred continuously for 15min, 30min, 1h, 2h, 4h and 16h. The supernatants were dialysed against water, then 40% (w/v) methanol and then water before being freeze-dried. Insoluble material was washed extensively with water and freeze-dried. After being weighed carefully, all supernatants and pellets were hydrolysed in acid and assayed for hydroxyproline as described below.

Preparation and homogenization of muscle samples

Bovine pectoralis profundis muscle was removed whole from the carcases of 18month animals by careful dissection. Epimysium, which appeared as an obvious sheath or partial surface connective tissue, was carefully cut away with a scalpel, with as little adherent muscle as possible. Epimysium was washed briefly in phosphate-buffered saline (0·15M-NaCl/0·02M-sodium phosphate buffer, pH7.4) and stored frozen at −20°C.

The remaining muscle was sampled such that a strip weighing approx. 100g representing the general aspects of the whole muscle was taken. This was then cut into 1cm cubes, and 50g was homogenized, in 100ml of ice-cold 0.05M-$CaCl_2$ for 10s at full speed in a Waring Blendor. The homogenate was filtered through a graded copper grid with 1mm square holes, and then the second 50g was similarly homogenized and filtered. The combined materials not passing through the filter were then rehomogenized in a further 100ml of ice-cold 0.05M-$CaCl_2$ and were re-filtered. This process was repeated a further two times, at which point all the filtrates were combined, as was the retained gross connective tissue. This latter fraction was referred to as the perimysial fraction, and the filtered material was denoted the endomysial fraction.

Further extraction of muscle perimysium

Water-washed samples of the perimysial fraction, when reduced with KB^3H_4 (see below) and analysed for collagen cross-links, gave very poor profiles on ion-exchange chromatography. Similarly, when these preparations were digested with CNBr, peptide maps of inferior quality were always obtained after SDS/polyacrylamide-gel electrophoresis. Consequently, this fraction was subjected to three SDS washes as described for

lung tissue by Laurent *et al.* (1981). The lumpy, aggregated, perimysium was blot-dried on Whatman 3MM chromatography paper and was frozen in small pieces in liquid N_2. It was then powdered to approx. 100μm pieces in liquid N_2 in a Spex Freezer Mill (Glen Creston, Stanmore, Middx., U.K.). The powder was then extracted three times in 1% (w/v) SDS for 30min at room temperature, with the insoluble material being collected by centrifugation between each wash. The supernatants were combined, dialysed against distilled water, 40% (v/v) methanol and then distilled water, and then freeze-dried. The pellet was washed in water exhaustively, dialysed against 40% (v/v) methanol and was re-washed in water before further treatments.

Preparation of endomysium

The endomysial fraction from the homogenization was treated as described by McCollester (1962). A loose precipitate formed at 4°C when the suspension was left overnight. The supernatant, which contained no endomysial sheaths as assessed by light microscopy, was aspirated off and discarded. The pellet was resuspended in 25mM-NaCl containing 2.5mM-DL-histidine adjusted to pH7.4 with 1M-Tris and stirred in this buffer for 5min. The suspension was centrifuged for 300*g*-min and the supernatant was discarded. This procedure was repeated three times, and then the suspension was incubated in the same buffer at 37°C for 30min before a further five washes. The final pellet was resuspended in distilled water adjusted to pH 7.5 with 1M-Tris and washed for 10min before centrifugation at 300*g* for 15min. The endomysial preparation was then either extracted in 1% (w/v) SDS as described above and used for determination of type I and III collagen content and cross-link content or was extracted with a modified Hasselbach–Schneider buffer for the preparation of native collagens. This latter treatment involved two extractions of the pellet with a buffer containing 0.5M-KI, 40mM-$Na_2B_4O_7$, 10mM-$Na_2P_2O_7$ and 10mM-NaH_2PO_4, pH8.8. The suspension was finally left being stirred overnight in the buffer at 4°C, during which time the collagen formed a fibrous knot around the magnetic stirrer bar. The supernatant was carefully decanted and reserved, and the fibrous material was collected, blot-dried and weighed. Approx. 25g of this material was obtained per kg of fresh muscle. After a brief wash in water, the material was ready for pepsin digestion and was termed endomysium preparation A. The reserved supernatant, which contained some endomysial sheaths on examination in the phase-contrast microscope, was centrifuged at 300*g* for 30min and the pellet was termed endomysium preparation B (wet weight approx. 2.5g).

Preparation of native collagen types

Washed perimysium or purified endomysium were suspended in 0.5M-acetic acid, and pepsin (Sigma Chemical Co.) was added to each to a final concentration equivalent to 1:100 enzyme to wet weight of substrate and the suspensions were incubated at 15°C for 24h. After digestion, insoluble material was removed by centrifugation and was found to account for 15% of the initial dry weight of the perimysium and 10–15% of the material in the endomysial preparations. Each pepsin-solubilized fraction was made 7% (w/v) in NaCl, and the resulting precipitate was collected. Neither 7%-salt supernatants from perimysium or endomysium contained any collagenous protein by SDS/polyacrylamide-gel-electrophoretic analysis and so were discarded. As both endomysium preparations A and B appeared to be identical by electrophoretic analysis, they were combined.

Precipitated pepsin-solubilized collagen from both perimysial and endomysial preparations was then redissolved in 0.2M-NaCl/0.02M-Tris-HCl buffer, pH7.4, and was subjected to salt fractionation by the method described by Dixit *et al.* (1981).

CNBr digestion and SDS/polyacrylamide-gel electrophoresis

Perimysium that had been extracted with 1% (w/v) SDS was blot-dried and weighed, and powdered in the freezer mill. The product and the final endomysial pellet were suspended in 70% (v/v) formic acid to a concentration of 50mg/ml, and an equivalent of one-fifth the wet weight of each of CNBr dissolved in acetonitrile (2g/ml) was added. Incubation was carried out at 30°C for 4h, at which time samples were rotary-evaporated after 10-fold dilution and then freeze-dried.

CNBr-cleavage peptides were separated on SDS/10% (w/v) polyacrylamide gels as previously described (Light, 1982) by the method of Laemmli (1971). Whole collagen molecules were separated on 5.5% (w/v) polyacrylamide gels as described by Sykes & Bailey (1971). Gels were stained with Coomassie Brilliant Blue and destained in 10% (v/v) methanol/7.5% (v/v) acetic acid.

Cross-link analysis and hydroxyproline estimation

SDS-washed samples were suspended in phosphate-buffered saline at room temperature and were reduced with KH^3B_4 as previously described (Robins *et al.*, 1973). After 30min acetic acid was added to adjust the pH to 4.0, and the samples were

washed free of soluble radioactivity. Samples were then freeze-dried and hydrolysed in 6M-HCl at 110°C for 24h under reflux. Hydrolysates were rotary-evaporated to dryness, and then taken up in 0.1M-pyridine/formate buffer, pH2.9, and subjected to ion-exchange chromatography on Zeolit 225 in pyridine/formate buffers as previously described (Light & Bailey, 1982). Cross-link peaks were identified and quantified by scintillation counting of radioactivity.

Hydroxyproline was analysed by a high-pressure-liquid chromatographic procedure modified from the method described by Umagat *et al.* (1982). Hydroxyproline in acid hydrolysates of SDS-washed perimysial and endomysial samples was treated with the fluorescent secondary-amine reagent 7-chloro-4-nitrobenz-1-oxa-1,3-diazole (2mg/ml in methanol) by the following method. Samples were diluted so that they contained approx. 3–4 μmol of collagen (0.5 μmol of hydroxyproline)/ml, and 0.2ml was added to 0.2ml of 0.5M-sodium borate buffer, pH9.5, and then 0.2ml of 7-chloro-4-nitrobenz-1-oxa-1,3-diazole reagent was added. After incubation at 60°C for 5min, samples were rapidly cooled to 4°C, then immediately analysed by high-pressure liquid chromatography. This was done on a 25cm × 0.46cm Du Pont Zorbax ODS 5 μm column at room temperature in a Du Pont Series 8800 liquid chromatograph with a flow rate of 1ml/min. Solvent A was 0.05M-sodium acetate buffer, pH6.6, containing tetrahydrofuran (1%, v/v), and solvent B was 90% (v/v) methanol. Hydroxyproline was eluted with a gradient of 33–43% solvent B over 6min, and the column was regenerated by washing with 100% solvent B for 4min and re-equilibrating at 33% solvent B for 10min.

Electron microscopy

Perimysial and endomysial fractions were examined by electron microscopy for measurement of fibre diameter after embedding, sectioning and positive staining as described by Causton (1980). Rotary shadowing of molecules was done by the method of Kühn *et al.* (1981). Bovine endomysial type IV collagen was dissolved at 0.5mg/ml concentration in 0.05M-acetic acid, and an equal volume of glycerol was added with thorough mixing.

The solutions were sprayed from an atomizer on to freshly cleaved mica sheets and air-dried in the evacuated chamber of an Edwards coating unit (model E306A). Molecules were shadowed with platinum at an angle of 9° and then carbon at 90°. Carbon replicas thus made were floated on to water, collected on copper grids and examined in a Philips EM 400 electron microscope.

Immunological assessment of endomysial type IV collagen

Antibodies raised in rabbits against foetal bovine skin type IV collagen and human placenta type IV collagen were purified and rendered type-specific as previously described (Bailey *et al.*, 1979*a*). Enzyme-linked immunosorbent assays of bovine endomysial preparations of type IV collagen were carried out with the use of coatings of 500ng, 200ng and 100ng per well as previously described (Black *et al.*, 1983). Inhibition assays were carried out by coating micro titre plates with 500ng of bovine endomysial type IV collagen/well and incubating optimal concentrations of antibody with doubling dilutions of both bovine and human type IV collagens before standard enzyme-linked immunosorbent assay.

Results

Solubility of aldimine-cross-linked collagen in SDS

Rat tail tendon washed in 1% (w/v) SDS solutions showed negligible swelling in 1h, although swelling was apparent from 2h. By 16h most of the collagen appeared in the swollen state and the supernatant was opaque. Analysis of the supernatants showed that 4% (dry weight) of the total material had been solubilized after 1h at room temperature, although only 17% of this was collagen. By 16h 75% of the tendon had been solubilized, and of this 77% was collagen. In short, only 0.7% of the total collagen content was solubilized by SDS washing at room temperature for 1h, and so we adopted three 30min washes as a routine for muscle connective tissue. In our hands the 1% (w/v) SDS solution had a pH of 5.3, and this mild acidity alone could account for the overnight solubilization of aldimine (acid-labile)-cross-linked collagen. By neutralizing the solution with Tris/HCl buffer the solvent could be used for more extensive (24h) washing with little or no solubilization of aldimine-cross-linked collagen.

Yields and electron microscopy of muscle connective-tissue preparations

Table 1 shows the yields of epimysium, perimysium and endomysium that we obtained from pectoralis profundis muscle. Calculation of the total muscle collagen content by summation of all three gave a value of 4.88% of the dry weight of the muscle. From Dransfield's (1977) value of 4.94% this gives a recovery of 98.8%, although our own estimation of muscle collagen (see below) was a minimum of 5.2%, lowering the recovery to 94%. When we checked the recovery of total muscle collagen after preparation of epimysium, perimysium and endomysium from four other muscles we obtained values ranging from 60% to 90%.

Losses tended to be greater in muscles with much lower collagen contents, but with muscles such as pectoralis profundis, gastrocnemius and sternomandibularis we always recovered greater than 75% of the total muscle collagen (N. D. Light & A. E. Champion, unpublished work).

Examination in the electron microscope of perimysial and endomysial connective tissues prepared as described in the Materials and methods section showed a convincing separation of the perimysium (which contains fibres of large diameter) described by Rowe (1978) from the endomysia and their related smaller fibres. The perimysial fibres were fairly uniform in cross-section and qualitatively larger than those seen in the endomysial preparations.

Collagen types isolated from muscle connective tissue

Fig. 1 shows the results of SDS/polyacrylamide-gel-electrophoretic analysis of pepsin-solubilized

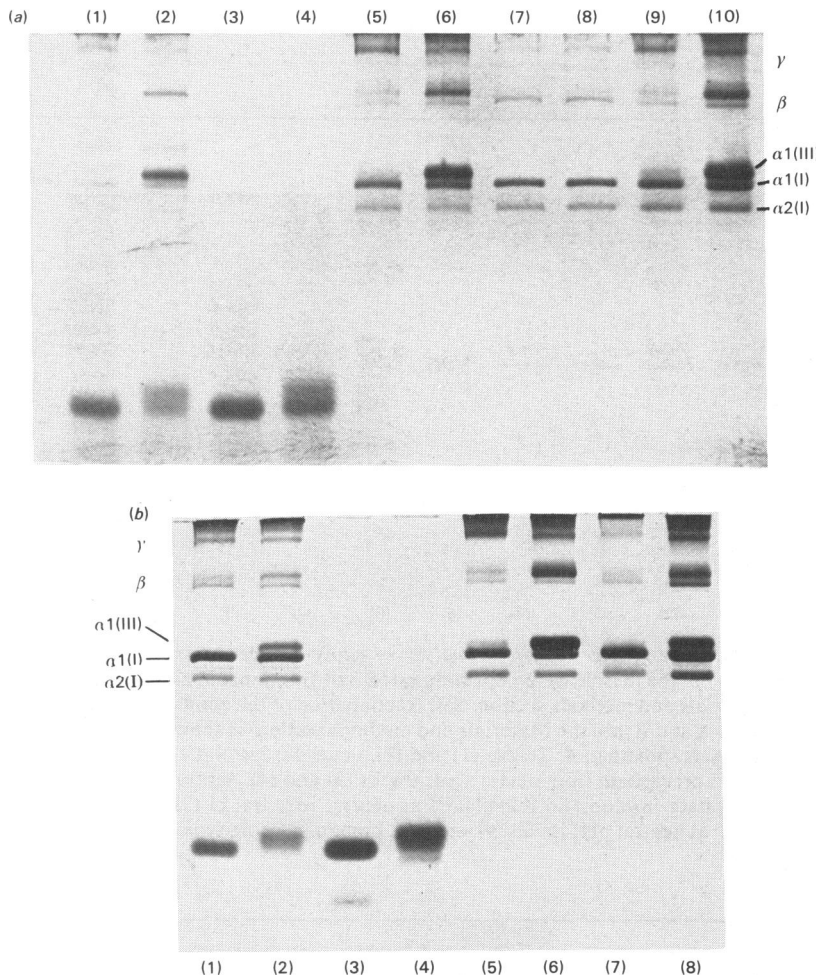

Fig. 1. *SDS/polyacrylamide-gel-electrophoretic analysis of native collagens prepared from muscle epimysium and perimysium* Purified epimysium and perimysium from bovine muscle were digested with pepsin, and the native collagens released were fractionated by salt precipitation and analysed by SDS/polyacrylamide-gel electrophoresis as described in the Materials and methods section. (*a*) Epimysium peptic digest. Tracks (2), (4), (6), (8) and (10) were run in the presence of added 2-mercaptoethanol. Tracks (1) and (2), pepsin-insoluble material (21% of total); tracks (3) and (4), 7%-NaCl supernatant from 0.5M-acetic acid (residual pepsin); tracks (5) and (6), neutral-1M-NaCl precipitate; tracks (7) and (8), neutral-1M-NaCl supernatant; tracks (9) and (10), 2M-NaCl precipitate insoluble in 0.2M-NaCl at neutral pH. (*b*) Perimysium peptic digest. Tracks (2), (4), (6) and (8) were run in the presence of added 2-mercaptoethanol. Tracks (1) and (2), pepsin-insoluble material (15% of total); tracks (3) and (4), 7%-NaCl supernatant from 0.5M-acetic acid (residual pepsin); tracks (5) and (6), 1M-NaCl precipitate; tracks (7) and (8), 2M-NaCl precipitate insoluble in 0.2M-NaCl at neutral pH.

Table 1. *Content of total collagen, type I and type III collagen and reducible cross-links in the connective tissues of bovine pectoralis profundis muscle*

Collagen content of connective tissue was assessed from hydroxyproline assays of SDS-washed connective tissue.

Muscle connective tissue	Connective tissue content of muscle (% of total dry wt.)	Collagen content of connective tissue (%)	Type III collagen content (% of type III + type I collagen)	Dihydroxylysinonorleucine/ hydroxylysinonorleucine ratio
Epimysium	1.2	22.2	16.4	2.5
Perimysium	4.7	95.3	28.0	2.5
Endomysium	0.3	42.4	62.3	6.0
Total recovery	6.2	4.88	–	–
Total muscle collagen	–	5.2 (4.94*)	–	–

* From Dransfield (1977).

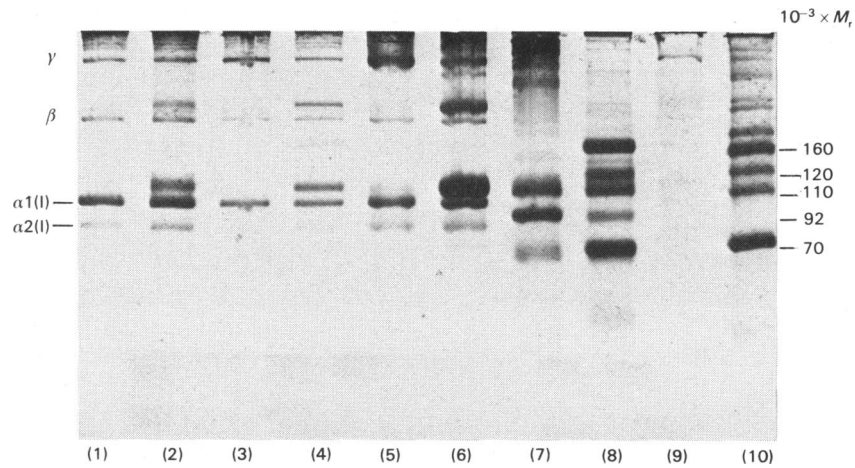

Fig. 2. *SDS/polyacrylamide-gel-electrophoretic analysis of native collagens prepared from muscle endomysium*
Purified endomysia from bovine muscle were pepsin-digested, salt-fractionated and analysed by gel electrophoresis as described in the Materials and methods section. Salt fractionation of the combined pepsin-soluble proteins from endomysial preparations A and B (see the Materials and methods section) is shown. Tracks (2), (4), (6), (8) and (10) were run with added 2-mercaptoethanol. Tracks (1) and (2), neutral-0.2M-NaCl-insoluble material obtained after redissolution of 7%-NaCl precipitate from acetic acid; tracks (3) and (4), neutral-2M-NaCl precipitate; tracks (5) and (6), 2M-NaCl precipitate insoluble in 0.2M-NaCl at neutral pH; tracks (7) and (8), neutral-2M-NaCl pellet redissolved in 0.2M-NaCl at neutral pH; tracks (9) and (10), human placenta type IV collagen prepared as described by Bailey *et al.* (1979*c*).

salt-fractionated collagens from epimysium and perimysium preparations. The predominant form of collagen present in both connective tissues is type I, but in both cases there is evidence of the other major fibrous form, type III collagen. A small and variable amount of type V collagen was present in some perimysial preparations, but represented a very minor component of this connective tissue.

Fig. 2 shows the final salt precipitates and supernatants from the endomysial preparations. As can be seen, both type I and III collagens were present in large amounts and accounted (with the very small amount of type V collagen seen in the final precipitate) for 95% of the recovered pepsin-soluble collagen. Type IV collagen, accounting for the final 5% of solubilized collagen, appeared as a clean preparation consisting largely of the three major bands at M_r 160000, 100000 and 70000 previously observed (Bailey *et al.*, 1979*c*), with an additional reducible chain of M_r 120000 and a partially reducible chain of M_r 92000. These extra

products were probably due to excessive pepsin treatment and further fragmentation of parent molecules.

Electron microscopy of endomysial type IV collagen

Fig. 3 shows a micrograph of a typical field seen in the electron microscope after rotary-shadowing endomysial type IV collagen with platinum. The molecules appear as a range of species with some full tetramers ('spiders') and many partially cleaved tetramers, both exhibiting the 7S domain, and pepsin-cleaved helical domain (Timpl *et al.*, 1981). There is little evidence of the NC1 region, which appears to have been fully cleaved under the pepsin degradation conditions chosen.

Immunological identity of purified endomysial type IV collagen

When purified human placenta type IV collagen was assayed by enzyme-linked immunosorbent assay against antibodies to human type IV collagen and antibodies to bovine type IV, it reacted strongly with both. However, bovine endomysial type IV collagen reacted only with antibodies to bovine type IV collagen and not with the antibodies to human type IV collagen. Inhibition enzyme-linked immunosorbent assay corroborated this evidence, and confirmed the immunological identity of bovine endomysial type IV collagen with bovine foetal skin type IV collagen against which the antibodies to bovine type IV collagen were raised.

Relative content of type I and III collagens

Fig. 4 shows a polyacrylamide-gel electrophoretogram of the CNBr-cleavage peptides obtained from SDS-washed and unwashed epimysium, perimysium and endomysium. The predominant peptides are from types I and III collagen, with little evidence of peptides from types IV or V collagens. SDS washing, as reported by Laurent *et al.* (1981), results in much cleaner gels and quantifiable peptide bands. The percentage of type III collagen (as a function of total type I + type III collagen), quantified from peptides α1(III) CB5 and α1(I) CB8 as indicated, was 16.4%, 28.0% and 62.3% in epimysium, perimysium and endomysium respectively (Table 1).

Hydroxyproline and reducible cross-link content of epimysium, perimysium and endomysium

Selected pieces of muscle, representative of the whole muscle mass, were chopped, homogenized and SDS-washed as described in the Materials and methods section. This method, complete within 2 days, allowed hydrolysis of a clean gross connective-tissue product that gave unequivocal analysis of hydroxyproline by the high-pressure-liquid-

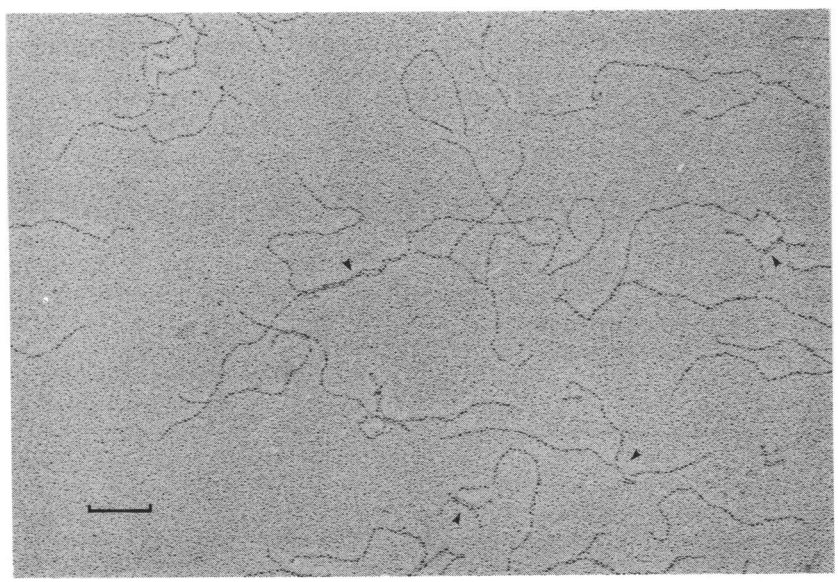

Fig. 3. *Electron microscopy of rotary-shadowed bovine endomysium type IV collagen*
Purified pepsin-solubilized bovine endomysium type IV collagen (Fig. 2, tracks 7 and 8) was rotary-shadowed with platinum and carbon as described in the Materials and methods section and viewed in the electron microscope. The field shows extensive degradation of type IV tetramers by pepsin (7S domains denoted by arrows). The bar is equivalent to 100 nm.

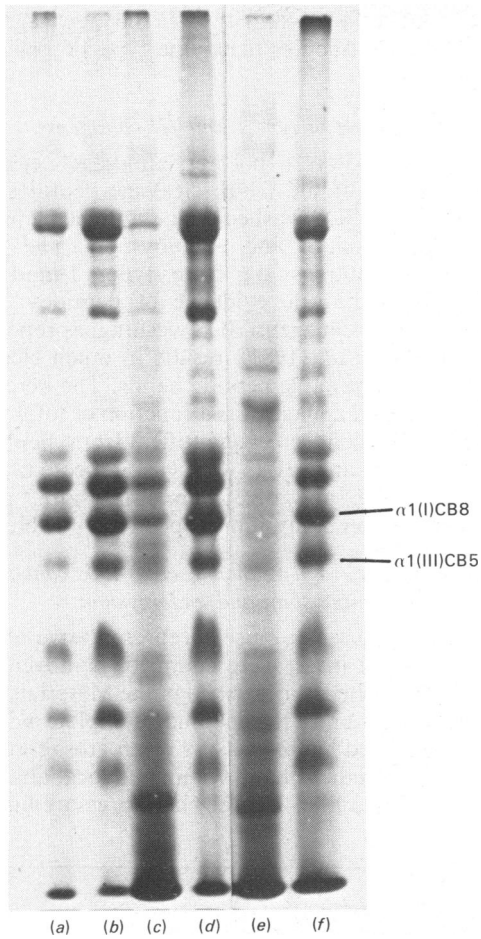

Fig. 4. *SDS/polyacrylamide-gel electrophoresis of CNBr-cleavage peptides of unwashed and SDS-washed bovine muscle epimysium, perimysium and endomysium*

Unwashed and SDS-washed muscle connective-tissue fractions were digested with CNBr and analysed by gel electrophoresis as described in the Materials and methods section. Tracks (*a*), (*c*) and (*e*) represent CNBr-cleavage peptides from raw preparations of epimysium, perimysium and endomysium respectively. Tracks (*b*), (*d*) and (*f*) represent mixtures of CNBr-cleavage peptides from SDS-washed epimysium, perimysium and endomysium respectively.

chromatographic method. Two pieces of pectoralis profundis, cut from the ends of the muscle at the tendon insert (wet weights 100 and 800mg), contained $18.1 \pm 0.8\%$ and $13.7 \pm 0.5\%$ collagen of the dry weight, whereas a piece weighing 2800mg from the central portion of the muscle contained only $5.2 \pm 0.1\%$ collagen of the dry weight. In this way the total hydroxyproline and therefore collagen content of any piece of muscle could be calculated, assuming a mean hydroxyproline content of 14%, without interference from chromogenic factors known to be present in muscle (Etherington & Sims, 1981).

When the three types of muscle connective tissue were reduced with tritiated borohydride after being washed only with salt, relatively poor cross-link analyses were obtained after acid hydrolysis (for example see Fig. 5*a*). However, when the samples were extracted with SDS before reduction and cross-link analysis, much clearer profiles were obtained, as illustrated in Fig. 5(*b*). We found that the epimysia, perimysia and endomysia contained a mixture of the aldimine and ketoimine cross-links (hydroxylysinonorleucine and dihydroxylysinonorleucine respectively) in various proportions (Table 1).

Discussion

Because of the need to investigate muscle connective tissue at the molecular level, several groups of workers have attempted to devise simple methods of purifying this ubiquitous but minor component of the muscle mass (McCollester, 1962; McClain, 1969; Sanes & Hall, 1979; Fujii & Murota, 1982). Although methods for the isolation of endomysial (muscle-cell basement membrane) 'ghosts' have proved successful (McCollester, 1962; Sanes & Hall, 1979), the remaining muscle connective tissue was discarded. In other cases muscle connective tissue was prepared in bulk and treated as one undifferentiated component (McClain, 1969; Fujii & Murota, 1982).

Rowe (1978) comments on the paucity of micro-anatomical data on muscle connective tissue, but, in a brief microscopical study, supports Hamm's (1965) nomenclature of epimysium, perimysium and endomysium to describe the three main distinct muscle connective-tissue compartments. His data show that the epimysium, perimysium and endomysium are relatively discrete and distinguishable connective-tissue tracts that should be separated preparatively for biochemical analysis.

Our studies have shown that, by careful filtration and washing procedures after the homogenization process described by McCollester (1962), the bulk of the perimysium can be separated from the endomysial segments and their associated finer fibres. We chose to use Rowe's (1978) fibre size definition as a standard in our electron-microscope survey of the different connective-tissue pools that we obtained. Quantification of fibre diameter by using the digitizing pad on an Apple IIe computer confirmed the efficiency of the preparative method described here for perimysium and endomysium (N. D.

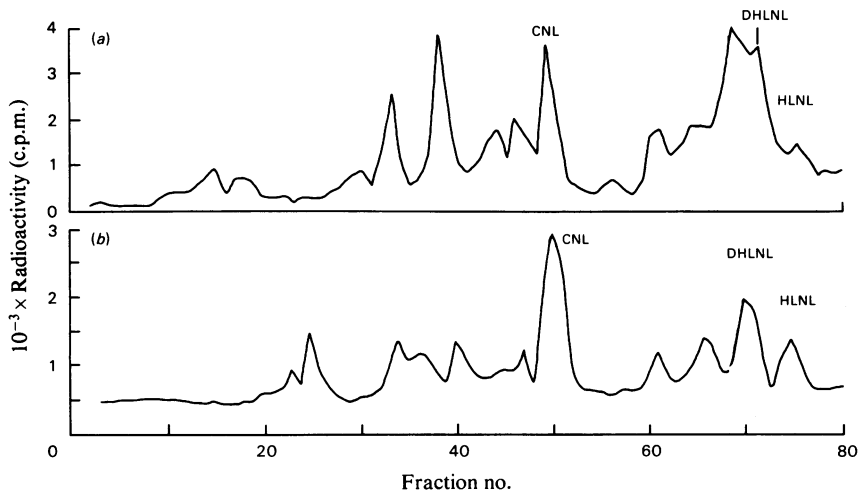

Fig. 5. *Analysis of cross-link content in muscle perimysium before and after SDS extraction*
Bovine muscle perimysium was prepared and analysed for cross-link content both before (*a*) and after (*b*) washing with 1% (w/v) SDS by the procedures described in the Materials and methods section. Key: CNL, chloronorleucine; DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine.

Light, A. E. Champion & C. Voyle, unpublished work).

By using our method with five different muscles we obtained high yields of connective tissue and, by quantifying the collagen content of each, were able to demonstrate recoveries of 60–90%.

Pepsin solubilization of our epimysium, perimysium and endomysium preparations allowed qualitative determination of the types of collagen present in these three connective-tissue compartments. We confirmed biochemically previous reports by Duance and co-workers (Bailey *et al.*, 1979*a*, *b*; Duance *et al.*, 1980*a*,*b*), who defined the distribution of types I, III, IV and V collagen in muscle by immunohistology. In our hands, epimysium contained only types I and III collagen, with the former being the most abundant. Bailey *et al.* (1979*a*) reported variations from little to no type III in a variety of muscle epimysia, a finding that is not contrary to our results as we found differences in epimysial type III collagen content throughout a range of muscles (N. D. Light, A. E. Champion & C. Voyle, unpublished work).

Our findings also support the immunological findings that type I collagen is the major component of perimysium, with approx. 30% type III and some associated type V collagen. We also showed that all four major collagen types may be found in endomysium. Undoubtedly, the type I and III collagen in the latter case are to be located in the so-called 'reticular' fibres associated with the muscle-fibre basement membrane, whereas the type IV is located in the basement membrane itself.

By using the simple methods reported here combined with washing with SDS solutions, as suggested by Laurent *et al.* (1981) for lung preparations, we have been able to produce extremely clean insoluble collagen preparations for hydroxyproline, cross-link and quantitative type I and type III collagen analyses. Control experiments with rat tail tendon type I collagen, which is entirely cross-linked by the acid-labile aldimine dihydrohydroxylysinonorleucine, showed that SDS solutions may be used with impunity for washing for periods of 1–2h at room temperature. Under such conditions less than 5% of aldimine-cross-linked collagen is solubilized, and, if neutralized, these solutions may be used for extensive washing of more intractable tissue without further solubilization of collagen. Addition of dithiothreitol or 2-mercaptoethanol at 5mM concentration was found to increase the efficacy of such washing procedures.

In the present paper we also describe the first isolation of type IV collagen from muscle basement membrane. It is now generally agreed that the basic molecular structure of type IV collagens isolated from such diverse basement membranes as glomerulus, lens capsule, placenta, EHS sarcoma and Descemet's membrane is very similar if not identical (Bornstein & Sage, 1980). Our results show that muscle basement-membrane type IV collagen is also very similar to other type IV collagens prevously described. It has the typical three chains with M_r 160000, 100000 and 70000, as shown by SDS/polyacrylamide-gel-electrophoretic analysis (see Bailey *et al.*, 1979*c*), and the unique

'spider'-like structure, when viewed by electron microscopy, consisting of the 7S domain and four helical 'legs'. It seems reasonable to assume that the intact molecules also contain NC1 globular regions (Timpl *et al.*, 1981), but that these were lost by pepsin cleavage during preparation. Finally, the endomysial type IV collagen prepared here was immunologically identical with foetal bovine skin type IV collagen, as assessed by enzyme-linked immunosorbent assay with antibodies raised against the latter collagen. Endomysial type IV collagen showed a low degree of cross-reactivity with antibodies raised against human placental type IV collagen, showing few shared antigenicities between the two molecules.

In conclusion, we have described a rapid and efficient preparative procedure, based on several earlier methods, for the production of all three major connective-tissue tracts of muscle. This procedure should prove useful in the further study at the molecular level of the role of collagen in muscle connective tissue and in the investigation of abnormalities occurring in various pathological conditions in the different levels of muscle connective tissue.

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