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Connective tissue changes in immobilised muscle

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INTRODUCTION

The loss of sarcomeres and reduction in muscle fibre length which occurs when muscles are working at a shortened length (Williams & Goldspink, 1973) is associated with an increased resistance to passive stretch. Length/tension curves for muscles immobilised in a shortened position are steeper than those for control muscles (Williams & Goldspink, 1978) and this is the case even when allowances are made for the shorter lengths of the immobilised muscles (Goldspink & Williams, 1979).

The forces passively imposed on a muscle by stretching are distributed over the tissue as a whole by means of the intramuscular connective tissue framework (Hill, 1950; Winegrad & Robinson, 1978). The amount and arrangement of connective tissue in the perimysium and endomysium would be expected to affect muscle elasticity. It was decided, therefore, to see whether the increased stiffness of immobilised muscle could be accounted for by an increase in collagen content and whether any increase was mainly in the endomysium or in the perimysium. It was thought to be of particular interest to determine the time course of any changes since connective tissue accumulation could result either directly from the immobilisation procedure or from a redistribution of connective tissue following the loss of sarcomeres and shortening of the fibres.

MATERIALS AND METHODS

The mice used in these experiments were normal heterozygous 8 weeks old males of the strain 129 Re. One hind limb of each animal was immobilised by means of plaster of Paris bandage so that the soleus muscle was held in the shortened position. After periods ranging from one day to four weeks the animals were killed and the soleus muscles removed. A few muscles were immobilised for two weeks in the lengthened position.

Biochemical assay

Muscles which had been immobilised for periods ranging from five days to four weeks were used. Only the middle section of each muscle was included, i.e. that portion which contained no tendon tissue. Since the muscle pieces were very small they were pooled into groups of four before being weighed, freeze dried and weighed again. The dry tissue was dissolved in 6 N-HCl and hydrolysed by autoclaving. Hydroxyproline was determined using a technicon autoanalyser following the method of Grant (1964). Since hydroxyproline can be considered to occur exclusively in collagen, collagen content may be calculated from the hydroxyproline

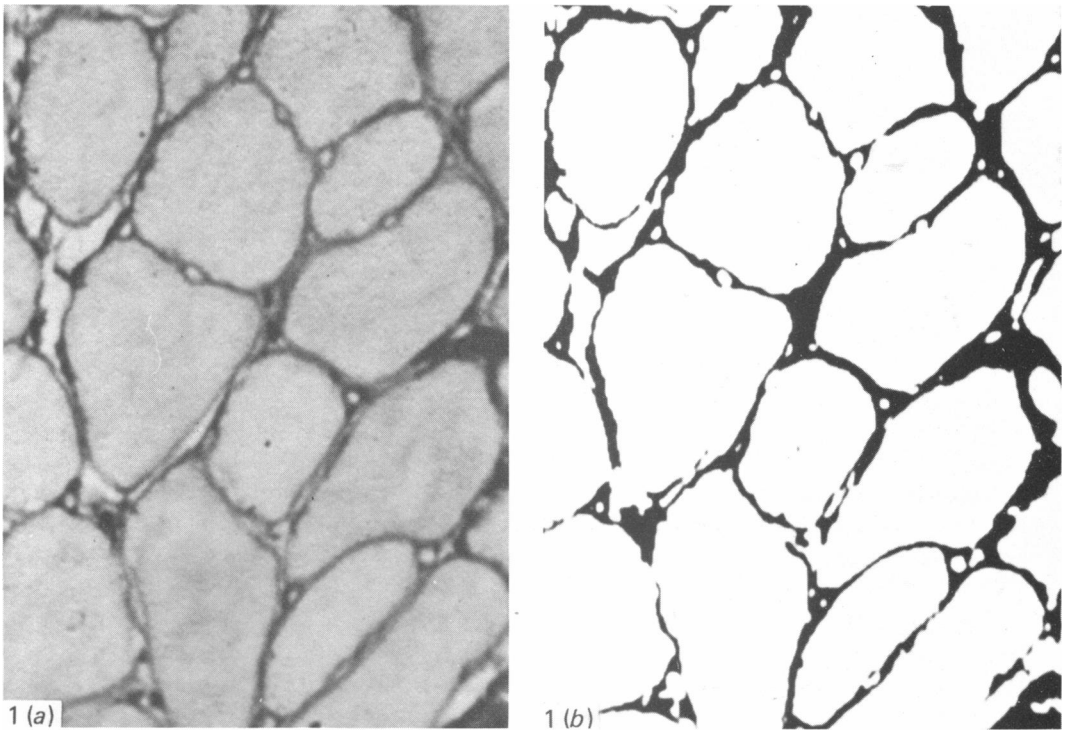

Fig. 1 (*a–b*). Mouse muscle stained for connective tissue with Sirius Red F3 BA: (*a*) optical image after passing through a green filter. (*b*) Video image displayed on a monitor. A variable threshold facility removes grey gradation so that the image is a mixture of black or white points. An electric circuit allows the blanking of the image so that a small area of known dimensions can be studied. Logic circuits count the number of prints in any one frame. ×900.

content of the hydrolysate (Jackson & Clearey, 1967). The analysis was carried out on three groups of muscles for each period of immobilisation.

Sarcomere number measurements

The method used to determine the number of sarcomeres along the length of single fibres has been described in detail previously (Williams & Goldspink, 1971). After varying periods of immobilisation, the animals were killed and the soleus muscles fixed in 2·5 % glutaraldehyde. The muscles were then placed in 30 % HNO_3 to hydrolyse the connective tissue and stored in 50 % glycerol. Individual whole fibres were teased out and mounted. Using a projection microscope, the number of sarcomeres in series was counted. Three measurements were made on each muscle and three muscles used for each period of immobilisation.

Image analysis

After periods of immobilisation ranging from one day to three weeks, soleus muscles were frozen in isopentane and then sectioned at a thickness of 10 μm in a cryostat. Sections from the mid-belly region were stained for connective tissue using Sirius Red F3BA (Sweat, Putchler, Sanford & Rosenthal, 1964). This dye stained the connective tissue bright red which, under a green optical filter, appeared black and contrasted well with the pale muscle fibres. The sections were examined under a

Leitz microscope to which was attached an image analyser consisting of a video system linked to a small computer. Using this system, muscle sections were scanned selecting a small area of known dimensions and a reading taken which represented the connective tissue content.

Using a control slide, the video image was adjusted by means of a threshold facility so that there was good correspondence between the optical and the video images (Fig. 1*a*, *b*). No further adjustments were made whilst taking readings of slides from all the experimental and control muscles for a given period of immobilisation: sections from these muscles were stained under identical conditions and the single setting proved satisfactory for all the slides. No adjustments were made to take account of slight differences in section thickness, since it was assumed that any such variation would be similar in experimental and control muscles. A control slide was included in each batch of stained slides and was analysed after adjusting the threshold. Since there was no significant difference between readings taken from different control slides, it was possible to compare directly the results from the various experimental groups.

Changes in endomysial thickness were estimated by examining areas of the muscle cross section which contained no perimysium, whilst the amount of perimysial connective tissue was deduced by scanning the entire muscle section, i.e. using areas which contained both perimysium and endomysium. Five experimental and five control muscles were scanned for each period of immobilisation and 10–15 readings taken from each muscle. In the case of the endomysial connective tissue analysis the number of fibres in each area was counted.

One section from each muscle belly was stained with a Mallory–Heidenhain stain for muscle fibres. Using a low magnification, the total cross sectional area of the muscle was determined. In this way, the total connective tissue per muscle cross section could be estimated.

Fibre diameter measurements

Frozen cross sections from the mid-belly region of each experimental and control muscle were examined with a Leitz Ortholux microscope, the eyepiece of which contained graduated cross-hairs. Two estimates were made of the diameter of a fibre and the average value was recorded. The graduations on the cross-hairs were calibrated using a stage micrometer. The muscle section was scanned from the deeper surface to the outer surface a number of times: as the slide was moved across the stage, each fibre touched by the centre of the cross-hairs was measured; 100 readings were taken from each muscle.

Ultrastructural analysis

Orientation of collagen fibres in the perimysium was studied by scanning electron microscopy. Muscles which had been immobilised in the shortened position for two weeks were fixed, in the same position, with 5 % phosphate-buffered glutaraldehyde, pH 7·4, and then washed in buffer. Control muscles were fixed in either the lengthened or shortened position. A bundle of fibres from the mid-region of each muscle was then dissected, post-fixed in 2 % phosphate-buffered osmium tetroxide, dehydrated in a graded series of acetones and subjected to critical point drying using carbon dioxide. Samples were mounted on specimen stubs, coated with gold and examined in the scanning electron microscope.

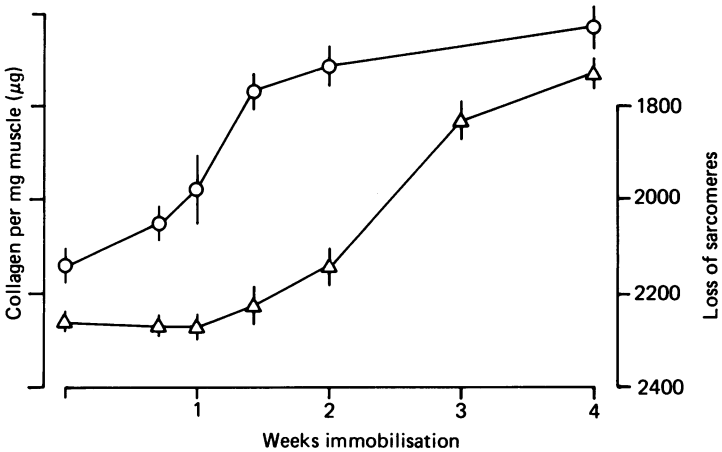

Fig. 2. Graph showing the relationship between loss of sarcomeres in series (△) and hydroxyproline content (○) of muscles immobilised for different lengths of time. Each point represents the mean ± standard error of three readings.

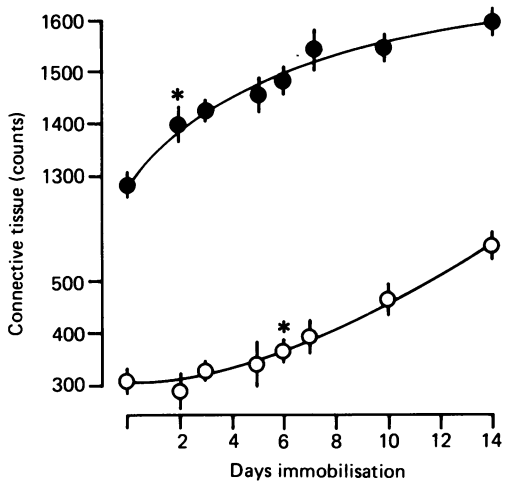

Fig. 3. Image analysis of connective tissue content of immobilised muscles. Cross sections of the muscle belly were scanned selecting, first, areas which contained only endomysium (○) and, second, using a larger frame size, areas which contained both perimysium and endomysium (●). Each point represents mean ± standard error of 50–75 readings. *, Threshold at which there is a significant difference between experimental and control measurement ($P < 0.01$).

RESULTS

Biochemical assay

Analysis of hydroxyproline content in the immobilised muscles showed that there was a considerable increase in collagen concentration (Fig. 2) and that this increase occurred during the very early stages of immobilisation.

Image analysis

Image analysis also demonstrated an increase in the ratio of connective tissue to muscle fibre tissue in immobilised muscles (Fig. 3). After a two weeks period of immobilisation there was, however, a decrease in the total amount of connective

Table 1. *The effect of immobilisation of the mouse soleus muscle on fibre diameter, fibre packing and loss of connective tissue and muscle fibre tissue*

	Control	Immobilised			
		2 days	7 days	10 days	14 days
Fibre diameter ± S.E.* (μm) (n=500)	46·3±0·7	46·9±0·8	40·7±0·6	35·7±0·7	31·2±0·9
Number of fibres per frame (n=50)	6·0	6·3	7·5	8·6	9·9
Counts (endomysium) per fibre (n=50)	52	47	54	55	57
Loss of connective tissue per cross sectional area (%) (n=5)	—	—	14	18	22
Loss of muscle fibre tissue per cross sectional area (%) (n=5)	—	—	19	25	39

* Standard error.

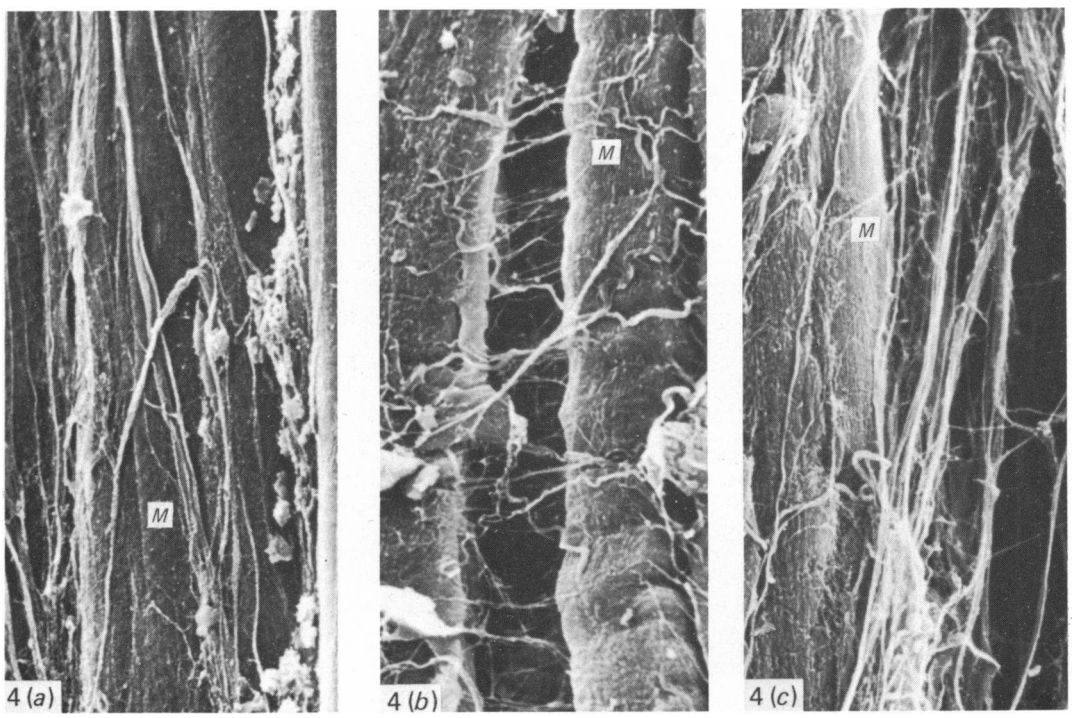

Fig. 4 (*a–c*). Scanning electron micrographs of collagen fibres in the perimysium: (*a*) normal muscle fixed in the lengthened position. (*b*) Normal muscle fixed in the shortened position. (*c*) Muscle immobilised for two weeks in the shortened position and then fixed in the same position. *M*, muscle fibre. × 1300.

tissue per whole muscle cross sectional area (Table 1), the increased concentration resulting from a greater loss of muscle fibre tissue (Table 1).

The scans which included both endomysium and perimysium showed that an increase in connective tissue concentration occurred after only two days of immobilisation (Fig. 3). Scans which contained only endomysium showed no such early increase; thus it would appear that at this stage the connective changes are confined to the perimysium.

After approximately one week of immobilisation, an increase in concentration of endomysium was observed. This could have been partly due to fibre atrophy, resulting in an increase in the number of fibres in each area scanned (Table 1) and an increased ratio of surface area (i.e. endomysium) to muscle fibre tissue. However, when the connective tissue readings were expressed as counts per fibre, the results were not lower in the immobilised muscles and since fibre diameter (and hence perimeter) was significantly reduced, the results indicated that the endomysium was thicker in immobilised muscles.

Ultrastructural analysis

Under the scanning electron microscope, the collagen of the perimysium was arranged as a loose weave of fibres at an angle to the long axis of the muscle fibres. When normal muscle was fixed in the lengthened position, the collagen fibres made an acute angle with the muscle fibres (Fig. 4*a*). When the muscle was fixed in the shortened position, this angle was much greater (Fig. 4*b*). However, in muscles which had been immobilised in the shortened position for two weeks, the collagen fibres became orientated at a much more acute angle to the myofibre axis (Fig. 4*c*), than in normal muscle fixed in the same position.

DISCUSSION

The relationship between the arrangement of connective tissue and the physiological properties of a muscle is not clearly understood. However, it is generally thought that muscle can be considered in terms of an active contractile element, a passive series elastic component which transmits the active force of contraction, and a parallel elastic component which both distributes the forces associated with passive stretch and maintains the relative position of the fibres (Hill, 1953; McLaughlin & Sonnenblick, 1974). The perimysium is considered to be a major component in the parallel elastic component (Borg & Caulfield, 1980).

The results described here show that changes in the passive tension properties of immobilised muscle are accompanied by changes in the connective tissue component of the muscle. The events which occur during the first few days of immobilisation are particularly interesting. An increase in the amount of perimysium occurs after only two days of immobilisation, i.e. before there is evidence of loss of serial sarcomeres (Figs. 2, 3). The implication is that immobilisation has a direct effect on the connective tissue rather than that the increase is due to a redistribution of connective tissue following the shortening of the fibres. When muscles are immobilised in the lengthened position, there is no increase in the proportion of connective tissue to muscle fibre tissue: thus, it seems that it is not reduced activity *per se* which results in the changes but that it is the position of the limb which is important.

During the early stages of immobilisation, there is no significant decrease in muscle fibre diameter: hence the increase in ratio of connective tissue to muscle fibre tissue

would appear to represent an increase in the total amount of connective tissue in the muscle cross section. However, immobilisation in a shortened position for a longer period results in muscle fibre atrophy (Eccles, 1941; Brooks, 1970) and, although the ratio of connective tissue to muscle fibre tissue continues to increase, the total connective tissue per cross section decreases, the increased concentration resulting from a faster rate of loss of muscle fibre tissue.

After one week of immobilisation there appears to be a thickening of the endomysium. It is not certain whether the endomysium is involved in the parallel elastic component. Borg & Caulfield (1980) consider that the endomysium, arranged as a network of fine fibres closely associated with the basal laminae, is likely to be mainly involved in the series elastic component but suggest that part of the endomysium, consisting of fine branched collagen fibres which connect adjacent muscle cells, might function in stress resistance. In heart muscle, these connectives are taut in relaxation and slack in contraction (Caulfield & Borg, 1979). In skeletal muscle, however, it is not certain whether these connective tissue elements should be considered as part of the endomysium or as fine branches of the perimysium (Rowe, 1981).

Ultrastructural changes in the connective tissue are being investigated further. In particular, it is necessary to determine whether changes in orientation of the collagen fibres merely reflect a realignment of the collagen fibres; this might be expected to accompany the increase in sarcomere length which occurs when immobilised muscles lose serial sarcomeres (Williams & Goldspink, 1978). If, when immobilised and control muscles are fixed at optimum length (i.e. when sarcomere lengths are equal), the orientation of the collagen fibres were to be the same in the two muscle groups, then the changes observed in the present study would not be expected to affect muscle compliance.

The experiments carried out here indicate that in muscles which have been immobilised in the shortened position there are quantitative and possibly qualitative alterations in the connective tissue which are likely to result in the reduced elasticity observed in immobilised muscles. Similar changes may occur whenever a muscle is working at a shortened length.

SUMMARY

The reduction in fibre length of muscles immobilised in a shortened position is accompanied by reduced compliance of the muscle. Since the intramuscular connective tissue framework distributes the forces passively imposed on a muscle by stretching, it was decided to investigate the amount and distribution of connective tissue in immobilised muscles.

Biochemical analysis of the hydroxyproline content of muscles immobilised in the shortened position for different periods of time showed an increase in the ratio of collagen to muscle fibre tissue. This occurred during the first few days of immobilisation, before there was any significant loss of sarcomeres. Thus the increase in connective tissue appeared to result directly from immobilisation rather than from redistribution of connective tissue, following shortening of the fibres.

A detailed histological analysis of muscle sections stained for connective tissue with Sirius Red showed that the early increase in connective tissue in immobilised muscles occurred in the perimysium rather than the endomysium, although after a longer period of immobilisation there was also a thickening of the endomysium.

Ultrastructural analysis of the perimysium in normal muscle showed that the angle the collagen fibres made with the muscle fibres changed with the state of stretch of the muscle; when the muscle was shortened, the angle was larger than when the muscle was lengthened. In immobilised muscle, collagen fibres were found to be arranged at a more acute angle to the axis of the muscle fibres than was found in normal muscle; this would be expected to affect the compliance of the muscle.

The experiments described indicate that the increased stiffness of immobilised muscles could result from both quantitative and qualitative changes in the connective tissue.

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REFERENCES

BORG, T. K. & CAULFIELD, J. B. (1980). Morphology of connective tissue in skeletal muscle. *Tissue and Cell* **12**, 197–207.

BROOKS, J. E. (1970). Disuse atrophy of muscle. *Archives of Neurology* **22**, 27–30.

CAULFIELD, J. B. & BORG, T. K. (1979). The collagen network of the heart. *Laboratory Investigation* **40**, 364–372.

ECCLES, J. C. (1941). Disuse atrophy of skeletal muscle. *Medical Journal of Australia* **2**, 160-164.

GOLDSPINK, G. & WILLIAMS, P. E. (1979). The nature of the increased passive resistance in muscle following immobilization of the mouse soleus muscle. *Journal of Physiology* **289**, 55*P*.

GRANT, R. A. (1964). Estimation of hydroxyproline by autoanalyser. *Journal of Clinical Pathology* **17**, 685–687.

HILL, A. V. (1950). The series elastic component of muscle. *Proceedings of the Royal Society, B* **137**, 273–280.

HILL, A. V. (1953). The mechanics of active muscle. *Proceedings of the Royal Society, B* **141**, 104–117.

JACKSON, D. S. & CLEAREY, E. G. (1967). The determination of collagen and elastin. *Methods of Biochemical Analysis* **15**, 25–72.

MCLAUGHLIN, R. J. & SONNENBLICK, E. H. (1974). Time behaviour of series elasticity in cardiac muscle. *Circulation Research* **34**, 798–811.

ROWE, R. W. D. (1981). Morphology of perimysial and endomysial connective tissue in skeletal muscle. *Tissue and Cell* **13**, 681–690.

SWEAT, F., PUTCHLER, H., SANFORD, I. & ROSENTHAL, A. (1964). Sirius Red F3BA as a stain for connective tissue. *Archives of Pathology* **78**, 69–72.

WILLIAMS, P. E. & GOLDSPINK, G. (1971). Longitudinal growth of striated muscle fibres. *Journal of Cell Science* **9**, 751–767.

WILLIAMS, P. E. & GOLDSPINK, G. (1973). The effect of immobilization on the longitudinal growth of striated muscle fibres. *Journal of Anatomy* **116**, 45–55.

WILLIAMS, P. E. & GOLDSPINK, G. (1978). Changes in sarcomere length and physiological properties in immobilized muscle. *Journal of Anatomy* **127**, 459–468.

WINEGRAD, S. & ROBINSON, T. F. (1978). Force generation among cells of the relaxing heart. *European Journal of Cardiology* **7**, 63–70.