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COMPARISON OF ENZYME ACTIVITIES AMONG SINGLE MUSCLE FIBRES WITHIN DEFINED MOTOR UNITS

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SUMMARY

1. Muscle fibres from single motor units of rat extensor digitorum longus were depleted of their glycogen by electrical stimulation and identified by the periodic acid-Schiff stain after treatment in a medium that selectively enhanced glycogen content in the non-depleted fibres.

2. Malate dehydrogenase (MDH) and fructose-1,6-diphosphatase (FDPase) activities were studied quantitatively in single dissected fibres of individual motor units and in fibres selected randomly from the same muscle.

3. In contrast to the large variability of MDH and FDPase in muscle fibres taken randomly, the muscle fibres from the same motor units had similar enzyme activities.

4. The resistance to fatigue of the motor units correlated well with the capacity of aerobic oxidative metabolism, as judged by the activity of MDH in the muscle fibres.

INTRODUCTION

The metabolic characteristics of mammalian skeletal muscle are believed to be regulated in some way by the activity pattern imposed upon the muscle by its motor nerve (Drahota & Gutmann, 1963; Bücher & Pette, 1965; Pette, Smith, Staudte & Vrbová, 1973). If this is the case then all the muscle fibres composing a mammalian motor unit should have identical metabolic characteristics. Qualitative evidence for the homogeneity of the motor unit has been provided with regard to certain enzymes of energy metabolism using histochemical techniques (Edström & Kugelberg, 1968; Burke, Levine, Tsairis & Zajac, 1973). Recent advances in quantitative microchemistry have made it possible to determine enzyme activities on segments of single dissected muscle fibres. From studies on single muscle fibres of rabbits and man, it became clear that a wide, continuous spectrum of enzyme activity levels exists within a fibre population, a heterogeneity that is not detected using conventional histochemical techniques (Spamer & Pette, 1977, 1979; Lowry, Kimmey, Felder, Chi, Kaiser, Passonneau, Kirk & Lowry, 1978). Even fibre groups that have a similar histochemical appearance show pronounced variations of enzyme activities (Spamer & Pette, 1977, 1979; Nemeth & Pette, 1980). These quantitative findings challenge the histochemical evidence of the homogeneity of muscle fibres from individual motor units, and they predict that it is possible that variability of enzyme activities might be detected

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among fibres of a motor unit if the high sensitivity of the microanalytical method is employed. To examine this possibility, the activities of malate dehydrogenase (MDH) and fructose-1,6-diphosphotase (FDPase) were measured quantitatively in single muscle fibres of individual motor units. Preliminary results of this study have been reported (Nemeth, Pette & Vrbová, 1980*a*, *b*).

METHODS

Rats (Sprague–Dawley) weighing 150–200 g were anaesthetized by I.P. injections of chloral hydrate (35 mg/100 g body wt.). The spinal cord was exposed by laminectomy and one of the ventral roots at the level of L4 was dissected. The extensor digitorum longus muscle was prepared for tension recording by freeing its distal tendon. The muscle was kept moist in oxygenated Krebs solution at 37 °C. The sciatic nerve was then exposed and, apart from the nerve to the extensor digitorum longus, all branches supplying other leg muscles were cut. The leg was held rigidly attached to a heavy steel plate. The tendon of EDL muscle was connected to a strain gauge and contractions were displayed simultaneously on a Devices pen recorder and the screen of an oscilloscope.

The exposed roots were washed with warm oxygenated Krebs–Henseleit solution, then submerged in liquid paraffin and split into small filaments. These were stimulated using fine silver–silver chloride wire electrodes. Filaments containing axons to single motor units gave all-or-none responses to low voltage electrical pulses, and this response did not increase with increasing stimulus intensity. The ventral root filaments were then stimulated by trains of 10 pulses at 40 Hz repeated every second, and the tension developed by the muscle in response to this stimulation was monitored.

Motor units ascertained to be sensitive to fatigue by a rapid and marked decrease in tension were stimulated for 30 min, while those units resistant to fatigue, showing a small and gradual decrease in tension, were stimulated for 60–90 min. It is known from previous studies that the susceptibility of muscle fibres to glycogen depletion is correlated to their susceptibility to fatigue (Edström & Kugelberg, 1968; Kugelberg & Edström, 1968; Burke *et al.* 1973). We found the relationship of glycogen depletion and fatigue to be variable. Stimulation was, in some trials, carried out for periods extending far beyond the time at which the lowest tension was reached.

Seven rapidly fatiguing units were stimulated for 60–90 min, and one fatigue-resistant unit was stimulated 120 min. While these units retained their low tension, their glycogen was not completely depleted. These findings indicated that resynthesis of glycogen may have taken place when stimulation continued for some time after the unit had been fatigued.

Glycogen depletion was most apparent in rapidly fatiguing units after 30 min of stimulation and in fatigue-resistant units after 60 min. These times were therefore used in the experiments.

Immediately following stimulation the muscle was quickly frozen in melting isopentane (−160 °C), then stored below −70 °C. From a piece of the muscle transverse sections of 20 μm were cut in a cryostat (−20 °C), and these sections were processed for the histochemical periodic acid-Schiff reaction for glycogen. Muscle fibres belonging to a stimulated motor unit were identified in these sections by their lack of staining (Kugelberg & Edström, 1968).

The muscles were then freeze-dried for 48 hr at −40 °C and a pressure less than 10^{-3} torr, and single fibres were isolated using the microdissection techniques of Essén, Jansson, Henriksson, Taylor & Saltin (1975). Fibres were dissected from the area which was identified by the histological section to contain the muscle fibres depleted of glycogen. A 100–300 μm segment of each dissected fibre was stained with periodic acid-Schiff reagent. It was necessary to enhance the contrast between depleted fibres and those non-depleted fibres containing low levels of glycogen, and this was accomplished by building-up their existing glycogen content by preincubating the fibre pieces in a reaction medium for glycogen synthetase (Pool, Donnelaar & Griep, 1978). Fibres depleted of glycogen particles are unable to further synthesize glycogen; this is probably due to the loss of glycogen synthetase along with other glycogen metabolizing enzymes which, in the absence of glycogen, become soluble and diffuse away during the histochemical incubation.

Plate 1 shows the contrast in PAS staining obtained after this preincubation between a glycogen depleted fibre and non-depleted control fibres.

TABLE 1. Enzyme activities (μ/g fresh wt.) in single fibres of rat extensor digitorum longus muscle (EDL)

EDL	Motor unit	Controls
	Malate dehydrogenase	
1	35·4– 38·4	52·7–156·2
2	101·8–106·8	45·2–119·8
3	99·9–102·7	35·9–120·3
4	47·9– 52·9	31·2–148·7
5	30·7– 32·3	20·2–135·8
6	75·8– 77·2	46·4–225·5
	Fructose-1,6-diphosphatase	
1	0·752–0·799	0·493–0·990

From the six muscles in which a single motor unit was depleted of its glycogen, over 3300 single fibres were dissected, catalogued and examined for glycogen depletion. From these, five to thirteen fibres of each motor unit were found, giving a total of forty-six fibres from six muscles.

Quantitative determinations for malate dehydrogenase (EC 1.1.1.37, MDH) and for fructose-1,6-diphosphatase (EC 3.1.3.11, FDPase) activities were made using the microchemical technique of Lowry & Passonneau (1972) on six to twelve pieces of each fibre from the identified motor units. Measurements were made concurrently on non-depleted fibres which had been randomly selected from the same muscle. The assay mixture for MDH contained 70·5 mM-triethanolamine-HCl, 7·05 mM-EDTA, 0·58 mM-NADH, bovine serum albumin 0·75 mg/ml. 0·5 mM-oxaloacetate, pH 7·6 (Spamer & Pette, 1977). The MDH activity was determined by measuring the NAD fluorescence in strong alkali, and calibrated by enzymatically measured NAD standards.

FDPase was measured according to Spamer & Pette (1979). The assay mixture contained 56 mM-imidazole, 1·1 mM-EDTA, 10 mM-$MgCl_2$, 0·42 mM-phosphoenolpyruvate, 0·015 mM-ATP, 0·3 mM-NADP, bovine serum albumin 0·75 mg/ml., glucose-6-phosphate dehydrogenase 26 μg/ml., phosphoglucose isomerase 26 μg/ml., adenylate kinase 15 μg/ml., pyruvate kinase 15 μg/ml., 125 mM-fructose-1,6-diphosphate, pH 7·4. The adenylate kinase and pyruvate kinase were included to remove tissue 5′AMP, a stronger inhibitor of FDPase.

The final product in the FDPase reaction, NADPH, was amplified by enzymatic cycling and measured by its native fluorescence (Lowry & Passonneau, 1972).

The activities of both enzymes were measured at 25 °C under conditions in which the formation of the reaction product was linear with time and with weight of fibre sample. Enzyme activities were expressed as μ/g fresh weight where $\mu = \mu\text{mole} \cdot \text{min}^{-1}$ (Spamer & Pette, 1977). As controls, photometrically measured assay reactions were run in parallel with micro-assays, using purified MDH or FDPase.

RESULTS

Enzyme activities. MDH activities in single randomly selected fibres of rat extensor digitorum longus muscles ranged between 20 and 226 μ/g fresh wt. (Table 1). The activities from different muscles were within the same range. While enzyme activity levels of the fibres from the six motor units ranged between 31 and 107 μ/g fresh wt. (Table 1), there was only a slight difference between different fibres of the same motor unit. The extent of the similarity of fibres from the same motor unit is apparent from Table 2. In fact, the variations of mean MDH activity between the fibres in each of the individual motor units was less than the variation of MDH activity between segments of the same fibre.

The FDPase activity in randomly selected fibres of one muscle ranged between 0·493 and 0·990 μ/g fresh wt. (Table 1). In contrast, FDPase activity of fibres

TABLE 2. Deviations in enzyme activities within motor units and within their single fibres. Coefficient of variation is the S.D. as a percentage of the mean

Motor unit	Number of fibres	Mean activity (μ/g fresh wt.)	Mean S.D. of motor unit	Coefficient of variation (%)	Mean S.D. of single fibres	Coefficient of variation (%)
			Malate dehydrogenase			
1	13	37·1	0·9	2·4	1·6	4·3
2	6	105·1	1·8	1·7	3·9	3·7
3	5	101·4	1·1	1·0	6·5	6·4
4	8	49·2	2·1	4·2	3·2	6·5
5	7	31·5	0·5	1·6	1·1	3·5
6	7	76·3	0·6	0·7	1·7	2·2
			Fructose-1,6-diphosphatase			
1	7	0·780	0·017	2·2	0·039	5·0

belonging to a single motor unit were very similar. The variation of activities of FDPase among fibres of the motor unit was, as in the case of MDH, less than that within pieces of single fibres.

Correlation of metabolic and physiological properties. While fibres of the same motor unit were identical, there was a considerable variability of MDH activity in fibres from different motor units. Two examples of motor units with very different MDH activities are shown in Fig. 1. This Figure also shows the decline of tension with time of the same two motor units. It is apparent from this Figure that the decline of tension was less in the motor unit that contained fibres with high MDH activities. Both of these motor units were fast contracting, with 20 and 23 msec time to peak twitch tension and 24 and 26 msec 1/2 time-to-peak tetanic tension. In this study fast contracting motor units that fatigued rapidly contained muscle fibres in which the activity of MDH varied within a narrow range of 32–49 μ/g fresh wt. In contrast muscle fibres from motor units that were resistant to fatigue had MDH activities of 101–105 μ/g fresh wt.

An index of fatigue after 30 min of continuous stimulation was assigned to each unit. In Fig. 2 this index is plotted against the MDH activities of fibres from each unit. The graph shows that the enzyme activities were proportional to the fatigue index, so that high MDH activities were associated with fatigue resistance.

DISCUSSION

Enzyme activities in muscle fibres from individual motor units were compared using high resolution microanalytical techniques. The results show that fibres belonging to the same motor unit are identical with regard to MDH and FDPase activities. The motor unit was identified by glycogen depletion achieved by prolonged stimulation of its motor axon. It is possible that a motor unit may contain some muscle fibres that are not depleted by the stimulation applied, and that these fibres are not identified as part of the motor unit, so that their enzyme activities would not be included in the sample. These fibres could be the ones that, in the fatigue test,

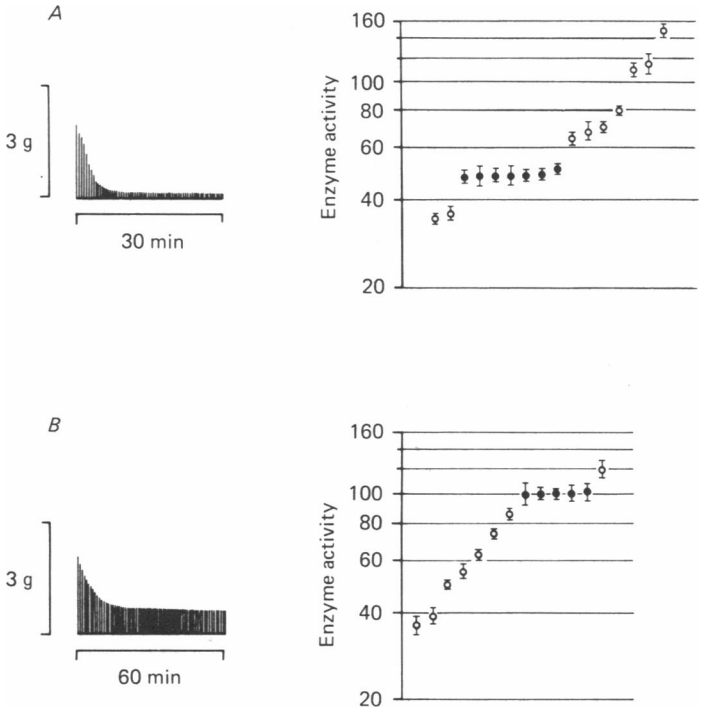

Fig. 1. Malate dehydrogenase content of fibres and fatigue characteristics of two motor units of rat extensor digitorum longus muscle. The mechanical records show the changes in maximal tetanic tension to repeated stimulation by trains of pulses every second, each train consisting of 10 pulses at a frequency of 40 Hz. MDH activities in single fibres of the motor unit (●) are distinguished from randomly selected fibres (○) of the same muscle. Ordinate: enzyme activity in μ/g fresh wt.; each circle represents the mean $\pm$ S.D. of six to twelve determinations on pieces of single fibres. The fibres are arranged in order of increasing activity. Note the logarithmic scale. Since there is no numerical magnitude for the abscissa, the graph does not represent a function.

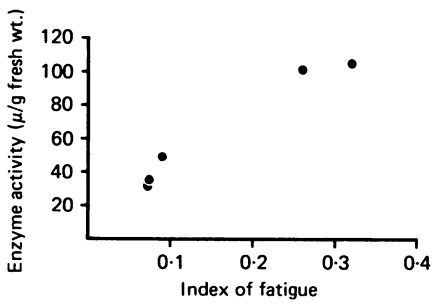

Fig. 2. Relationship between malate dehydrogenase activities in single fibres of fast-twitch motor units and their fatigue-resistance, measured as the fraction of initial tetanic tension remaining after 30 min of stimulation.

continue to contract after the initial steep drop in tension. However, one could expect that if such differences in susceptibility to fatigue and to glycogen depletion would exist the decrease in tension developed by the motor unit would continue with prolonged stimulation. The motor units in our study sustained their residual, small tension during the whole period of stimulation, so only a totally different small group of fibres could have escaped detection. Thus our results support the assumption that fibres belonging to the same motor unit are homogeneous.

Results from the recent study of Lowry *et al.* (1978) are also conistent with such an interpretation. These authors measured a variety of different enzymes within single fibres of muscles, and they found groups of fibres in which the activities of ten enzymes showed remarkable similarity between the fibres, while there was great variability between other groups of fibres from the same muscle. The present evidence would suggest that those fibres, whose enzyme profiles were the same belonged to the same motor unit.

The resistance to fatigue of individual motor units and its correlation with the MDH activities in their muscle fibres is in agreement with the findings of Edström & Kugelberg (1968) and Burke *et al.* (1973), based on histochemistry of oxidative enzymes. In the present study, resistance to fatigue correlated well with MDH activity; fibres with high levels of MDH fatigued less than those with low levels of MDH. No motor units with intermediate MDH activities, or fatigue indices were found. This failure to find intermediate units was probably due to the small sample of motor units studied since fibres with intermediate levels of enzyme activities were present among the randomly selected fibres.

Results from this study provide further support for the idea that control of the muscle fibre's metabolism is mediated by the specific neural input. Above all, by using this very high resolution technique, these results have revealed the remarkably precise control of the metabolic properties of muscle fibres exerted by the motoneurone.

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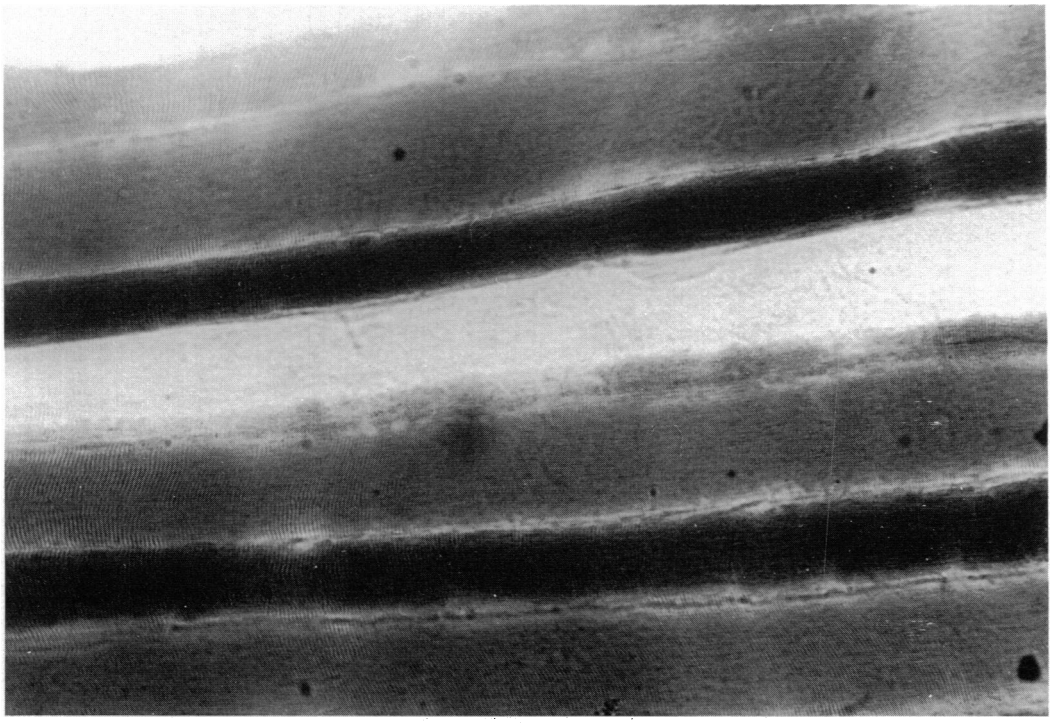

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EXPLANATION OF PLATE

Section of rat extensor digitorum longus muscle after fibres of a single motor unit were depleted of their glycogen. The tissue was preincubated in a reaction medium for glycogen synthetase in order to enhance the contrast in periodic acid-Schiff staining (for glycogen) between glycogen-depleted fibres, as illustrated in the centre of the field, and those non-depleted fibres having low glycogen content, such as the fibre just above the depleted one.