

SUCCINATE DEHYDROGENASE ACTIVITY IN FIBRES CLASSIFIED BY MYOSIN ATPase IN THREE HIND LIMB MUSCLES OF RAT

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SUMMARY

1. Succinate dehydrogenase (SDH) activity was assessed *in situ* in single fibres of cross-sectioned extensor hallucis longus, extensor digitorum longus, and soleus muscles of rat by means of microphotometric recordings of initial maximum reaction rates.

2. Each fibre assessed for SDH activity was subjectively classified into myosin subgroups by its histochemical reaction for myofibrillar actomyosin ATPase (myosin ATPase) following preincubation at pH 4.6 according to Brooke & Kaiser (1970).

3. The majority of fibres classified into myosin types I and IIa were highly reactive for SDH, such that those myosin groups could be interchangeable with the metabolic subgroups of Peter, Barnard, Edgerton, Gillespie & Stempel (1972); myosin I = slow-twitch oxidative, myosin IIa = fast-twitch oxidative glycolytic.

4. The myosin type IIb fibres, however, demonstrated marked variability in activity levels of SDH. Over 40% of those fibres had high SDH activity, and thus could not be equated with the metabolic subgroup fast-twitch glycolytic.

5. The histochemical reaction for myosin ATPase in muscle fibres therefore cannot be used as a reliable means to predict the fibres' metabolic characteristics.

INTRODUCTION

Muscle physiologists operationally classify fibres of mammalian skeletal muscle by using histochemical methods. The combination of histochemical reactions for myofibrillar actomyosin ATPase (myosin ATPase) and for enzymes of energy metabolism, gives rise to the fibre nomenclature slow-contracting oxidative (SO), fast-contracting oxidative glycolytic (FOG) and fast-contracting glycolytic (FG) of Peter *et al.* (1972). Another classification system, based on differential histochemical staining for myosin ATPase after preincubation at pH 4.6, gives rise to the types I, IIa and IIb of Brooke & Kaiser (1970).

After the introduction of these fibre types, several authors have inferred that the properties which the nomenclatures designate are correlated, as expressed in the review paper by Close (1972). The presumption was that myosin type I is equivalent to SO, myosin type IIa is FOG and type IIb is FG, although it was not directly shown. We decided to investigate whether the two systems of classification were

interchangeable. If the myosin ATPase reactivity does indeed indicate the fibres' metabolic properties, then a single histochemical parameter would certainly facilitate fibre typing. However, a false implication that the myosin type predicts the metabolic type might mislead the interpretation of physiological results.

We have provided evidence that myosin ATPase reactivity, after preincubation at pH 4.6, is not a reliable indication of the fibres' aerobic oxidative capacity, and, therefore, that the two systems of classification are not entirely interchangeable (Nemeth, Hofer & Pette, 1979; Nemeth & Pette, 1980a). In these previous studies, only qualitative histochemical methods were used. The visual assessment of final histochemical reaction products and the ensuing conclusions have been challenged (Spurway, 1981b). By using advanced microphotometric methods developed in our laboratory (Pette, Wasmund & Wimmer, 1979; Pette & Wimmer, 1979; Pette, 1981), it is now possible to quantitatively assess enzyme activities by initial rates of enzyme reactions in tissue sections. We have employed this technique to measure the succinate dehydrogenase (SDH) activity in individual fibres, classified qualitatively by myofibrillar actomyosin ATPase, in the extensor hallucis longus, extensor digitorum longus and soleus muscles of rat. SDH serves as a marker enzyme of mitochondrial aerobic substrate end-oxidation. Our findings confirm our previous conclusion that there is a great variety of oxidative enzyme levels in each of the myosin-based fibre categories. Therefore, there is no *a priori* justification for interchanging the two systems of classification.

METHODS

Adult male rats weighing *ca.* 250 g were used (strain Ch BB Thom FW 49, Thomae, Biberach, Germany). Animals had water *ad libitum* and were kept on a standard diet (Altromin, Altrogge, Lage/Lippe, Germany). Extensor hallucis longus (EHL), extensor digitorum longus (EDL), and soleus muscles were excised and quickly frozen in a slightly stretched position in melting isopentane (-160°C). Serial transverse sections ($10\ \mu\text{m}$) were cut at -25°C on a cryostat microtome (Fa. Dittes, Heidelberg, Germany). Tissue sections were alternately either stained for myosin ATPase after preincubation at pH 4.6 according to the methods of Brooke & Kaiser (1970), or prepared for microphotometric assays for succinate dehydrogenase (SDH).

The ATPase method was performed as follows: freshly cut sections were preincubated 10 min at room temperature in a 100 mM-Na acetate/acetic acid buffer, pH 4.60, containing 100 mM-KCl. After 30 sec in distilled water, sections were incubated 30 min at 37°C in a solution containing 3 mM-ATP and 30 mM- CaCl_2 and 50 mM-NaCl in a 50 mM-glycine/NaOH buffer adjusted to pH 9.6.

Sections were washed two times in distilled water, and were then incubated 3 min at room temperature in an aqueous solution of 150 mM- CoCl_2 . Thereafter sections were washed 3 times for 30 sec in distilled water and were then incubated 1 min in a 1% solution of $(\text{NH}_4)_2\text{S}$. After rinsing with distilled water, sections were dehydrated in ethanol and mounted in Entellan (E. Merck, Darmstadt, Germany). Solutions for preincubation at pH 4.6 and for incubation in the presence of ATP were made fresh. The pH was adjusted at room temperature using a glass electrode calibrated with fresh solutions of 'Titrisol' (E. Merck, Darmstadt, Germany) buffers (citrate/HCl, pH 2.0 and 4.0; phosphate, pH 7.0; borate/KCl-NaOH, pH 9.0).

For microphotometry, gel films (0.2 mm thick) containing the reaction medium for SDH were used (Nolte & Pette, 1972). The gel films contained 100 mM-phosphate buffer, 5 mM-ethylenediaminetetraacetic acid (EDTA), 1 mM, KCN, 0.2 mM-phenazine methosulphate, 50 mM-succinate, 1.5 mM-nitro-blue tetrazolium in 1.5% agarose (pH 7.6). In later experiments, gel films were replaced by an aqueous medium in which the agarose was omitted (Pette, 1981), providing identical results.

Microphotometric SDH activity determinations were made with the computer controlled LEITZ

MPV2 microscope photometer (Pette *et al.* 1979; Pette & Wimmer, 1979; Pette, 1981). Initial rates of enzyme reactions were measured at room temperature simultaneously in seven to eleven fibres selected at random. This was accomplished by automatically shifting the tissue position so that the selected fibres were moved successively into the measuring beam. A measuring field of $15 \times 15 \mu\text{m}$ was chosen and measurements were taken in the central region of each fibre in order to avoid subsarcolemmal mitochondria.

Measurements were made during continuous cycling among the selected fibres. One measuring cycle consisted of single measurements at each of the selected fibres and an area outside of the tissue section (control reaction). The time of a single measurement was 0.2 sec and successive measurements were taken at each fibre in fifteen measuring cycles. Total measuring time was 60–80 sec. By continually cycling among several fibres, the initial rate kinetics could be recorded simultaneously for the selected fibres. Enzyme activities ($\Delta E_{548} \cdot \text{sec}^{-1}$) were evaluated by the LEITZ LINREG computer subprogram (Pette *et al.* 1979), as maximum initial velocities.

Uncontrollable variations in section thickness, processing conditions and temperature are shown in previous studies to contribute to the variability of recorded absolute enzyme activities (Pette *et al.* 1979; Pette & Wimmer, 1979; Pette, 1981). By performing comparative activity measurements within the same section, such variations are eliminated.

A previous microphotometric study on the distribution of SDH activity along muscle fibres has made it possible to estimate the experimental error of this method (Pette, Wimmer & Nemeth, 1980). Comparative measurements along the same fibre gave extremely small differences (S.D. $\pm 4.6\%$). Assuming an even distribution of the enzyme along the fibres, this variation can be considered as representing the mean experimental error of the method.

In the present study, SDH determinations were performed on serial cross-sections. For each series of measurements, one fibre with low activity was selected as reference fibre from which to base relative activities. Its activity was set to equal 1 and all other activities were evaluated relative to it. The same fibre was identified in subsequent sections and assays. The resulting relative SDH activity eliminated variability due to tissue processing and conditions of the reaction.

The relative SDH activity obtained for each fibre was then paired with its myosin type according to the myosin ATPase reaction intensity found in the associated serial section. Myosin ATPase activity can not be measured as a direct microphotometric reaction because its final reaction product is formed after two coupled auxiliary reactions. The final staining product is clearly distinguishable into three groups, and easily assessed visually.

The EHL and EDL muscles were processed separately and the activities obtained for individual fibres are, therefore, compared among their own fibre population. In order to determine the relationship between the oxidative activity levels of fibres of EDL and soleus, these two muscles were processed together in a single block. By doing this, a single fibre could be used as reference for both muscles.

RESULTS

In 117 randomly selected fibres of the EHL muscle, the relative activity of SDH ranged from 0.63 to 4.78. Fibres identified as myosin type I by dark staining for myosin ATPase, preincubated at pH 4.6, had a range of relative SDH activity of 1.07–2.93. Myosin IIa fibres, staining lightly for preincubated myosin ATPase, had a range of 0.76–4.04. Myosin IIb fibres, staining intermediately for myosin ATPase, had a range of 0.63–4.78.

The relative SDH activities among the individual fibres of EHL muscle, and the distribution within myosin-based fibre groups, are seen in Fig. 1. The majority of myosin type I fibres have high relative SDH activity, over 1.5, as do most of the myosin type IIa fibres. This result is consistent with the view that myosin IIa fibres correspond to the metabolic type FOG, and the myosin I fibre corresponds to the metabolic type SO (Nemeth & Pette, 1980*a*, 1981; Spurway, 1980, 1981*a*, *b*). In the myosin type IIb group, 60% of the fibres have relative SDH activity below 1.5, and

could be considered as metabolic type FG. However, 40% of the myosin IIb fibres have SDH activity above 1.5. With respect to relative SDH activity in metabolic types SO and FOG, 40% of the myosin IIb fibres would be classified FOG.

The range of relative SDH activities in 60 fibres of EDL muscle is 0.51–6.12. Myosin type I fibres have an activity range of 2.88–3.48 (among only three fibres measured);

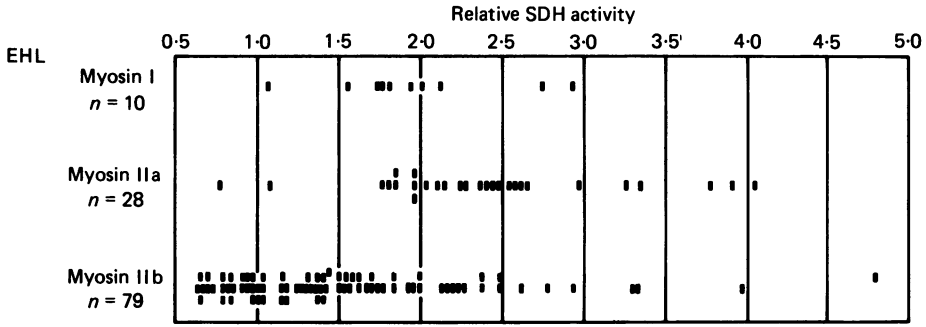


Fig. 1. Microphotometric assessment of relative SDH activity in single fibres selected in transverse sections of 4 extensor hallucis longus (EHL) muscles of two rats. Fibres were classified into three myosin ATPase groups following preincubation at pH 4.6 according to Brooke & Kaiser (1970).

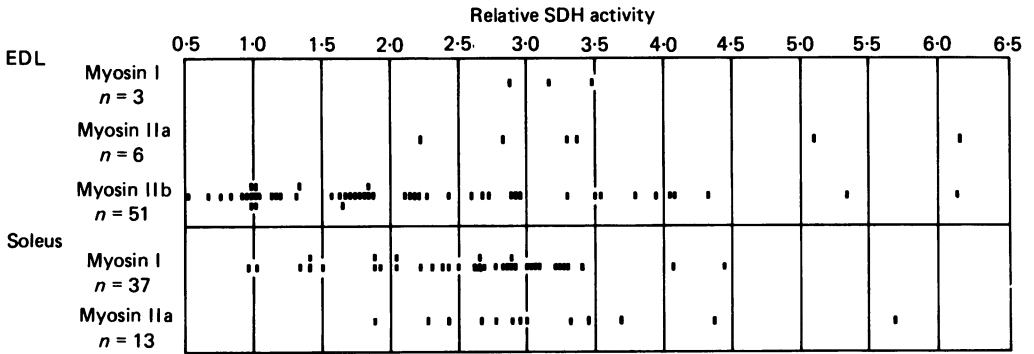


Fig. 2. Microphotometric assessment of relative SDH activity in single fibres selected in transverse sections of extensor digitorum longus (EDL) and soleus muscles of two rats. Fibres were classified into two or three myosin ATPase groups following preincubation at pH 4.6 according to Brooke & Kaiser (1970).

myosin IIa fibres have a range of 2.21–6.19 (only six fibres); and myosin IIb fibres have a range of 0.51–6.12. Fig. 2 gives the relative SDH activities of the individual fibres of the myosin groups.

The soleus muscle has only two myosin groups, indicated as I and IIa. The relative SDH distribution within the two myosin types is seen in Fig. 2. The myosin type I and IIa fibres overlap greatly; myosin I fibres range from 0.95 to 4.31, and myosin IIa fibres range from 1.88 to 5.68.

The same reference fibre for relative SDH activity was used for soleus and EDL muscle. Therefore, the fibre populations of the two muscles can be compared directly to each other. If the relative SDH activity in fibres of soleus muscle is used as the criterion for classifying fibres as aerobic oxidative, then the majority of the myosin II b fibres of EDL would be FOG.

DISCUSSION

Microphotometric recordings of initial reaction rates *in situ* reveal great variations in SDH activity among the individual fibres of EHL, EDL, and soleus muscles. These results are consistent with previous reports of extensive metabolic heterogeneity of muscle tissue. Metabolic heterogeneity has been best demonstrated by microanalytical biochemistry for a large number of enzymes on single dissected muscle fibres (Essén, Jansson, Henriksson, Taylor & Saltin, 1975; Spamer & Pette, 1977, 1979, 1980; Lowry, Kimmey, Felder, Chi, Kaiser, Passonneau, Kirk & Lowry, 1978; Lowry, Lowry, Chi, Hintz & Felder, 1980; Hintz, Lowry, Kaiser, McKee & Lowry, 1980). Even fibres with the same histochemical appearance show pronounced differences in absolute enzyme activities. Spamer & Pette (1980) have shown large differences in the activities of single type I fibres of soleus muscle of rabbit. However, this fibre population is relatively homogeneous with histochemical staining for oxidative enzymes. Histochemical distinctions may be even more obscure at high enzyme levels. We have recently reported (Nemeth & Pette, 1980*b*) that long term stimulation of the nerve to the tibialis anterior muscle of rabbit transforms the muscle from one having a variety of histochemical staining intensities for SDH in its fibres, to a strikingly homogeneous population of highly oxidative fibres. Microchemical analysis of the single fibres of that muscle, however, reveal a large range of the oxidative enzyme malate dehydrogenase. The range is only slightly less than the range of absolute activities in the heterogeneous population of normal tibialis anterior muscle. These studies provide strong evidence for great metabolic heterogeneity of muscle fibres, a heterogeneity far greater than that detected by conventional histochemistry. It may be that only fibres belonging to the same motor unit have truly identical enzyme characteristics (Nemeth, Pette & Vrbová, 1980, 1981).

Of critical importance to this discussion is the quantitated variability of metabolic activities in the myosin II b subgroup. In a recent study (Spamer & Pette, 1980) pieces of single dissected fibres of rabbit psoas muscle were stained histochemically for myosin ATPase, preincubated at pH 4.6, in order to select the myosin type II b fibres for study of their metabolic properties by quantitative microchemistry. The spectra of absolute activities for enzyme of both anaerobic and aerobic pathways in the myosin II b fibres were as great as the range of the enzymes in the entire muscle. Additionally, in that study, soleus type I fibres showed wide spectra of metabolic enzyme activities.

Using qualitative histochemical methods, large numbers of fibres from rat EHL and EDL muscles have been assessed for metabolic characteristics. Variations in enzyme reaction were found within the myosin fibre groups of each muscle (Nemeth, Hofer & Pette, 1979; Nemeth & Pette, 1980*a*). The present study utilizes advantages of two methods: quantitation of enzyme activities and measurements of large

numbers of fibres *in situ*. The results confirm our previous observation that there is marked overlap in enzyme activity levels among fibres of different myosin groups in these two fast-twitch muscles of the rat hind limb.

Despite fibre variability, simplified fibre classification systems may be applied. Metabolic and myosin profiles of individual fibres selected at random could be used to predict the muscles' fibre composition. Tables 1 and 2 were designed to compare

TABLE 1. Fibre composition of extensor hallucis longus muscle by two classification systems

A, classification based on myosin ATPase (without preincubation) and metabolic enzymes			B, classification based on myosin ATPase preincubated at pH 4.6		
SO	8	(9)	I	9	(10)
FOG	53	(54)	II a	23	(25)
FG	39	(37)	II b	68	(65)

Values expressed as % of a sample of fibres from a single muscle ($n = 117$) assessed by microphotometric recordings of initial rate reactions of SDH combined with qualitative histochemical reaction for myosin ATPase (A) and by qualitative histochemical reaction for myosin ATPase, preincubated at pH 4.6, alone (B). Fibres with relative SDH activity > 1.5 were classified as 'oxidative'.

Values in parentheses indicate % of three whole fibre populations ($n = 2019$) obtained by visual assessment of histochemical reactions (A) for SDH and myosin ATPase, and (B) for myosin ATPase preincubated at pH 4.6. Taken from Nemeth, Hofer & Pette (1979) and Nemeth & Pette (1980).

TABLE 2. Fibre composition of extensor digitorum longus muscle by two classification systems

A, classification based on myosin ATPase (without preincubation) and metabolic enzymes			B, classification based on myosin ATPase preincubated at pH 4.6		
SO	5 ^a	(5) ^b	I	5 ^a	(5) ^b
FOG	65	(57)	II a	11	(18)
FG	30	(36)	II b	84	(77)

See legend for Table 1. Numbers of fibres analysed was ^a60 and ^b669 (% less than 100 in column Ab indicates that some fibres did not fit within these categories).

previous data from qualitative studies with the present quantitative data on the same muscle. In order to do this, fibres characterized in the present study by relative SDH activity above 1.5 were designated 'oxidative'. Combined with their qualitative myosin ATPase reaction performed without preincubation, the fibres were referred to as either 'fast' or 'slow' (Bárány, 1967; Guth & Samaha, 1969). Glycogenolytic designation was applied to both of the myosin II subgroups based on our previous study (Nemeth *et al.* 1979). Microphotometric and qualitative histochemical means of assessing the metabolic characteristics of the fibre populations for simple fibre classification are in good agreement.

The muscle fibre composition obtained by using two classification systems can be compared in sections A and B of the Tables. Using either means of measuring metabolic characteristics, there is poor correlation between the myosin type II

subgroups and the metabolic subgroups. The II b subgroup is markedly heterogeneous with respect to aerobic oxidative capacity. The present microphotometric study supports previous qualitative estimates and provides strong evidence that the myosin-based classification system is not a reliable indicator of the metabolic characteristics of fast-twitch fibres.

That myosin type I and II a fibres nearly always utilize aerobic oxidation and that greater correlation of II b and FG fibres have been reported from other investigations (Spurway, 1980, 1981*a, b*) might suggest that properties of myosin and energy metabolism are somehow coupled. It is our point of view that if myosin properties and metabolism are indeed coupled, it might not be visualized by histochemistry. In the first place, myosin staining, depending on pH, provides two or three distinct fibre groups whereas even qualitative histochemistry gives a spectrum of oxidative levels. Also, it is not known what relationship the myosin ATPase stain has to properties of myosin (Brooke & Kaiser, 1970). Moreover, it is known that metabolic properties of muscle are highly mutable. Using chronic electrical stimulation, it has been shown that the time course of induced transformations in enzyme activities of energy metabolism are within days, while transformation of myosin properties require weeks or months (Pette, Smith, Staudte & Vrbová, 1973; Sréter, Gergely, Salmons & Romanul, 1973; Pette, Ramirez, Müller, Simon, Exner & Hildebrand, 1975; Pette, Müller, Leisner & Vrbová, 1976; Heilig & Pette, 1980). It has also been shown that the alterations of metabolic properties caused by exercise occur at different rates among the fibre groups, the myosin II fibres being most readily altered (Anderson & Henriksson, 1977; Holloszy, 1973). This mutability may account for the present finding that muscles have myosin II b fibres that are metabolically either FG or FOG, and it may explain discrepancies of fibre type correlations among different muscles, animals, or species.

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