

## **OXIDATIVE CAPACITY DISTRIBUTION IN SKELETAL MUSCLE FIBERS OF THE RAT**

MARIA PHILIPPI AND A. H. SILLAU

*Department of Physiology and Biophysics, School of Medicine, Medical Sciences  
Campus, University of Puerto Rico, GPO Box 5067, San Juan PR 00936, USA*

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### **Summary**

To study the distribution of oxidative capacity in muscle fibers, mitochondrial volume density and the oxidative capacity of isolated mitochondria were evaluated. Mitochondria were isolated from the subsarcolemmal and interfibrillar areas of the soleus (a muscle largely made up of slow oxidative fibers) and the gastrocnemius medial head (a muscle largely made up of fast glycolytic fibers) of the rat, and their oxidative capacities were evaluated using NADH- and FADH-generating substrates. In the soleus muscle, the subsarcolemmal mitochondria showed a lower oxidative capacity than interfibrillar mitochondria when NADH-generating substrates were used. This difference was not observed when FADH-generating substrates were used. In the gastrocnemius, there were no differences in the oxidative capacity of the subsarcolemmal and the interfibrillar mitochondria. Additionally, citrate synthase activity was found to be lower in mitochondria isolated from the subsarcolemmal area of the soleus than in the other mitochondrial preparations. These findings indicate that the difference in oxidative capacity of the isolated mitochondria is not related to differences in the inner mitochondrial membranes. Mitochondrial volume density was evaluated using electron micrographs of the subsarcolemmal and interfibrillar areas of slow oxidative fibers from the soleus and fast glycolytic fibers from the gastrocnemius. In the slow oxidative fibers, mitochondrial volume density in the subsarcolemmal area was four times higher than in the interfibrillar area. In the fast glycolytic fibers, mitochondrial volume densities in the subsarcolemmal and interfibrillar areas did not differ from that of the interfibrillar area of the slow oxidative fibers.

The oxidative capacity of the tissue, calculated by multiplying the mitochondrial oxidative capacities by the mitochondrial volume densities, was 2–4 times higher in the subsarcolemmal areas of the soleus fibers than in the other areas studied. This was true in spite of the fact that the oxidative capacity of the subsarcolemmal mitochondria of the slow oxidative fibers was lower than those of the other mitochondrial populations studied. These results indicate that the difference in oxidative capacity between slow oxidative fibers and fast glycolytic fibers is the result of the much greater mitochondrial volume density in the subsarcolemmal area of the slow oxidative fibers.

### **Introduction**

Three basic fiber types, each with considerable physiological and biochemical differences, are usually described in mammalian skeletal muscles. These fiber types are

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the slow oxidative (SO) fibers, the fast glycolytic-oxidative (FOG) fibers and the fast glycolytic (FG) fibers (Peter *et al.* 1972). SO and FOG fibers are thought to depend greatly on oxidative phosphorylation for their supply of ATP and to have substantially higher mitochondrial volume densities than the largely glycolytic FG fibers. Furthermore, the distribution of mitochondria is heterogeneous in oxidative fibers (SO and FOG), with mitochondrial volume density being higher in areas close to the membrane than in the core of the fibers. In contrast, in glycolytic fibers (FG), the distribution of mitochondria appears to be homogeneous (Eisenberg, 1983). In addition to this anatomical heterogeneity in the distribution of mitochondria in some fiber types, there is evidence that the oxidative capacity of the mitochondria is different in different regions of the muscle fiber. In heart muscle, Palmer *et al.* (1977, 1985) and McMillin-Wood *et al.* (1980) have described two populations of mitochondria with different oxidative characteristics. One of them, released by using the Polytron tissue processor (polytron mitochondria), is believed to be localized beneath the membrane and has a lower oxidative capacity than the other, which is associated with the myofibrils and is released after treatment of the remaining tissue with the proteolytic enzyme Nagarse (nagarse mitochondria). Because of their location within the fibers, polytron mitochondria are usually referred to as subsarcolemmal mitochondria, while nagarse mitochondria are referred to as interfibrillar mitochondria. Polytron and nagarse mitochondrial populations have also been isolated from the muscles of the hind limb of the rat by Krieger *et al.* (1980). As in heart muscle, these authors found that polytron mitochondria of skeletal muscle have a lower oxidative capacity than nagarse mitochondria. However, Krieger *et al.* (1980) used a muscle with a mixed fiber composition, and this precluded the possibility of determining whether the difference in oxidative capacity is a characteristic shared by the different fiber types or is limited to only some of them.

The oxidative capacity of a given volume of a cell is closely related to the product of the oxidative capacity of its mitochondria and its mitochondrial volume density. Thus, a better appreciation of the intracellular distribution of the oxidative capacity in skeletal myocytes can be obtained if information on these two variables is available. However, previous work has studied these variables individually. Data on the distribution of oxidative capacity within the muscles fibers are essential for producing better models of muscle oxygenation and for a better understanding of the physiological implications and significance of changes in oxidative capacity, such as those induced by exercise. To this end, we have studied the oxidative characteristics of polytron and nagarse mitochondria and measured mitochondrial volume density in an area close to the sarcolemma and in the core of the fiber. We have limited our studies to the SO fibers (soleus muscle) and to the FG fibers (white area of the medial head of the gastrocnemius muscle) mainly because no muscle or muscle area composed largely of FOG fibers could be found in the rat. The results of these studies are reported in the present paper.

## **Materials and methods**

### *Materials*

Sodium pentobarbital was purchased from Fort Dodge Laboratories (Iowa). Nagarse

was obtained from Enzyme Development Corporation (New York). Glutaraldehyde and  $\text{OsO}_4$  were purchased from Electron Microscopy Science (Pennsylvania). Lead citrate, uranyl acetate and Epon were purchased from E. F. Fullman Inc. (New York). All other chemicals were obtained from Sigma Chemical Co. (Missouri).

#### *Animals*

Female Wistar rats (Hilltop Laboratory Animals, Pennsylvania) weighing approximately 300 g were used in these experiments.

#### *Isolation of mitochondrial populations*

Animals were killed by decapitation and the soleus and the white area of the medial head of the gastrocnemius muscle (from now on referred to as gastrocnemius) were removed. Mitochondria were isolated as described by Krieger *et al.* (1980). Using this technique, two mitochondrial subpopulations are obtained from the muscle fibers. One of them was isolated after a short shock with a Polytron (Brinkman PT 10/35, Kinematica GmbH, Switzerland) (polytron mitochondria). The other was isolated after treatment of the remaining fraction of tissue with the proteolytic enzyme Nagarse (nagarse mitochondria).

Isolated mitochondria were resuspended in a medium containing (in  $\text{mmol l}^{-1}$ ): 220 mannitol, 70 sucrose, 5 Mops, 2 EGTA and 0.2 % bovine serum albumin (pH 7.4).

#### *Mitochondrial respiration*

Oxygen consumption of the mitochondrial fractions was measured with a Clark  $\text{O}_2$  electrode (Tomec and Hoppel, 1975) in a medium containing (in  $\text{mmol l}^{-1}$ ): 250 sucrose, 10 potassium phosphate and 1 Tris-HCl (pH 7.4) with 75–225  $\mu\text{mol l}^{-1}$  ADP (state III respiration) and after ADP exhaustion (state IV respiration). Three substrates were used: 20  $\text{mmol l}^{-1}$  glutamate and 2.5  $\text{mmol l}^{-1}$  malate; 10  $\text{mmol l}^{-1}$  pyruvate and 2.5  $\text{mmol l}^{-1}$  malate; and 10  $\text{mmol l}^{-1}$  succinate and 3.75  $\mu\text{mol l}^{-1}$  rotenone. The substrates pyruvate, glutamate and malate generate NADH that enters the mitochondrial respiratory chain at Complex I, while succinate in the presence of rotenone generates FADH that enters at Complex II. Respiratory control (RCR) and ADP/O (P/O) ratios were calculated in each case (Estabrook, 1967). Oxygen consumption was expressed as nanomoles per milligram of mitochondrial protein and per nanomole of cytochrome *c*+*c*<sub>1</sub>.

The activity of the enzyme citrate synthase was measured in the isolated mitochondria following the technique of Srere (1969). Mitochondrial protein was determined by the Lowry procedure with Folin and Ciocalteu's phenol reagent (Lowry *et al.* 1951). Cytochrome concentrations of the mitochondrial fractions were obtained as described by Williams (1964). Briefly, mitochondrial suspensions were solubilized with deoxycholate in two matched cuvettes. Potassium ferricyanide was added to one cuvette (oxidized) and sodium hydrosulfite to the other (reduced), the difference spectrum (reduced *versus* oxidized) was recorded and the concentrations of the cytochromes were then calculated from a set of simultaneous equations.

*Electron microscopy*

Five animals were anesthetized with sodium pentobarbital (50 mg kg<sup>-1</sup> body mass intraperitoneally) and their abdominal aortas were cannulated with a catheter that was advanced towards the hind limb. Heparin (500 i.u.) was injected through the catheter, the inferior vena cava was cut to allow perfusate outflow and perfusion was started through the aortic catheter with 50–70 ml of Lockes solution (pH 7.40) followed with 50 ml of 6.5 % glutaraldehyde in 0.1 mol l<sup>-1</sup> sodium cacodylate (pH 7.4). The temperature of the Lockes solution and the fixative was kept at 37 °C. Perfusion pressure was maintained at 13.3–16.0 kPa.

Small pieces (1 mm<sup>3</sup>) from the bellies of the fixed muscles were obtained, placed in fresh fixative and kept first for 1–4 h at room temperature and later at 4 °C for 18–36 h. The tissue was post-fixed in 1 % OsO<sub>4</sub>, dehydrated in acetone and flat-embedded in Epon. Silver to pale gold sections were obtained from blocks in which the fibers were found to be longitudinally oriented. The sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 transmission electron microscope. Photographs were taken at random with the only additional criterion that they show a part of the sarcolemma. Final magnification was 25 000×.

*Mitochondrial volume density*

Mitochondrial volume density was evaluated in the fiber core and in an outer annulus. This was a band limited by the sarcolemma and by a line traced 1 μm from the sarcolemma (Eisenberg, 1983). A grid of evenly spaced points, constructed following the recommendations of Weibel (1979a), was laid onto the electron micrograph and the points falling on the mitochondria in the core and the annulus were divided by the total number of points falling on each of these regions (Weibel, 1979a). In each muscle, the total area studied was approximately 2200 μm<sup>2</sup>.

**Results**

Polytron and nagarse mitochondrial suspensions from soleus and gastrocnemius showed a relatively high energy-conserving capacity and integrity. When using NADH-generating substrates, RCR values were between 4 and 9. These RCR values for polytron and nagarse mitochondria did not differ for a given muscle with the exception of the values for the soleus when pyruvate/malate was used as substrate. ADP/O values were approximately 2.9 and they did not differ for the different mitochondrial subpopulations.

In the presence of ADP (state III respiration), soleus polytron mitochondria oxidized NADH-generating substrates more slowly than the other mitochondrial subpopulations (Figs 1 and 2). However, no difference in the oxidation rate was observed when succinate/rotenone, which generates FADH, was used as substrate (Fig. 3). The results also show that gastrocnemius polytron and soleus and gastrocnemius nagarse mitochondria oxidize all the substrates tested at similar rates (Figs 1–3). Similar patterns were observed in the absence of ADP (state IV respiration). When the oxidation rates of the different mitochondrial subpopulations were expressed per nanomole of cytochrome *c+c<sub>1</sub>*, the same results were evident (data not shown). Cytochrome *c+c<sub>1</sub>* and *a+a<sub>3</sub>*

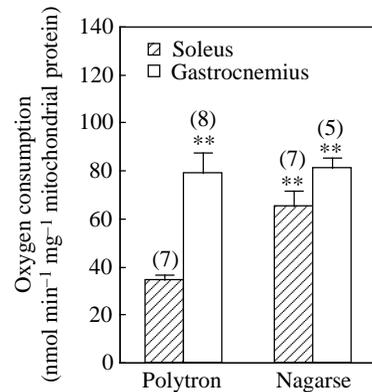


Fig. 1. State III respiratory rate of soleus and gastrocnemius mitochondrial subpopulations with glutamate/malate as substrate. The columns represent mean values. The bars indicate 1 S.E.M. Figures in parentheses are numbers of observations. \*\*Significantly different from soleus polytron mitochondria ( $P < 0.001$ ). Respiratory control ratios (RCR) for soleus polytron and nagarse mitochondria were  $8.6 \pm 1.3$  and  $9.3 \pm 1.3$  respectively. RCR values for gastrocnemius polytron and nagarse mitochondria were  $6.0 \pm 0.6$  and  $5.4 \pm 0.4$  respectively.

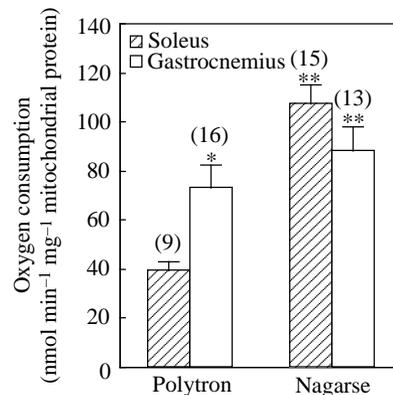


Fig. 2. State III respiratory rate of soleus and gastrocnemius mitochondrial subpopulations with pyruvate/malate as substrate. The columns represent mean values. The bars indicate 1 S.E.M. Figures in parentheses are numbers of observations. \*Significantly different from soleus polytron mitochondria ( $P < 0.01$ ). \*\*Significantly different from soleus polytron mitochondria ( $P < 0.001$ ). RCR values for soleus polytron and nagarse mitochondria were  $4.4 \pm 0.2$  and  $5.8 \pm 0.3$  respectively. RCR values for gastrocnemius polytron and nagarse mitochondria were  $5.0 \pm 0.3$  and  $5.0 \pm 0.5$  respectively.

concentrations were similar in the polytron and nagarse mitochondria of both muscles (Figs 4 and 5). Citrate synthase activity was lower for the soleus polytron mitochondria than for the other mitochondrial subpopulations (Fig. 6).

The electron micrographs from the soleus were of SO fibers and those of the gastrocnemius were of FG fibers. This conclusion was based on the width of the Z band

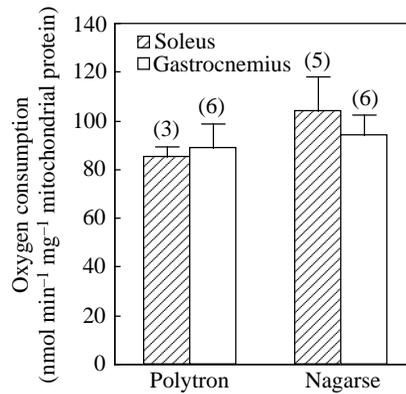


Fig. 3. State III respiratory rate of soleus and gastrocnemius mitochondrial subpopulations with succinate/rotenone as substrate. The columns represent mean values. The bars indicate 1 S.E.M. Figures in parentheses are numbers of observations. RCR values for soleus polytron and nagarse mitochondria were  $2.6 \pm 0.1$  and  $2.8 \pm 0.2$  respectively. RCR values for gastrocnemius polytron and nagarse mitochondria were  $3.0 \pm 0.1$  and  $3.8 \pm 0.2$  respectively.

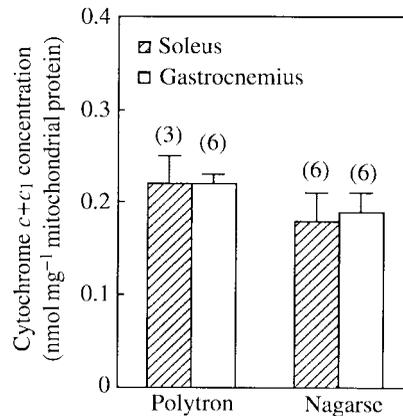


Fig. 4. Cytochrome  $c+c_1$  concentration in polytron and nagarse mitochondria isolated from the soleus and gastrocnemius muscles. The columns represent mean values. The bars indicate 1 S.E.M. Figures in parentheses are numbers of observations.

and the abundance of the mitochondrial profiles. In the annulus (subsarcolemmal) area of the soleus muscle fibers, mitochondrial volume density was much greater than in the corresponding area of the gastrocnemius muscle fibers or in the core (interfibrillar) areas of either muscle fibers (Fig. 7). Values of mitochondrial volume density in the annulus and core of the gastrocnemius muscle fibers were not significantly different from each other or from the value for the core of the soleus muscle fibers (Fig. 7).

### Discussion

Our results on the oxidative capacity of the mitochondrial subpopulations of the soleus

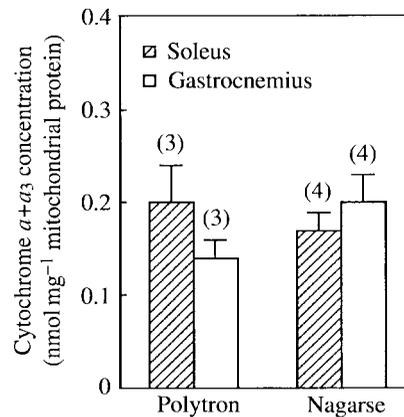


Fig. 5. Cytochrome  $a+a_3$  concentration in polytron and nagarse mitochondria isolated from the soleus and gastrocnemius muscles. The columns represent mean values. The bars indicate 1 S.E.M. Figures in parentheses are numbers of observations.

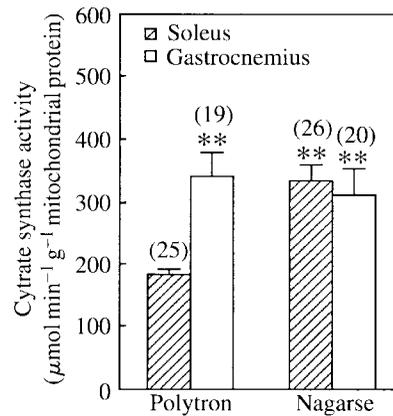


Fig. 6. Citrate synthase activity in polytron and nagarse mitochondria from the soleus and gastrocnemius muscles. The columns represent mean values. The bars indicate 1 S.E.M. Figures in parentheses are numbers of observations. \*\*Significantly different from soleus polytron mitochondria ( $P < 0.001$ ).

(Figs 1 and 2) are in agreement with results previously reported for heart muscle by Palmer *et al.* (1977) when NADH-generating substrates were used. However, in contrast to what has been reported for heart muscle (Palmer *et al.* 1977), we found that there were no differences in oxidative capacity between soleus polytron and nagarse mitochondria when an FADH-generating substrate was used (Fig. 3). Similarly, in the gastrocnemius, the oxidative capacities of polytron and nagarse mitochondria did not differ when NADH- or FADH-generating substrates were used (Figs 1–3).

Krieger *et al.* (1980) studied the oxidative capacity of polytron and nagarse mitochondria isolated from the gastrocnemius muscle and found that polytron mitochondria showed a lower oxidative capacity than nagarse mitochondria. They,

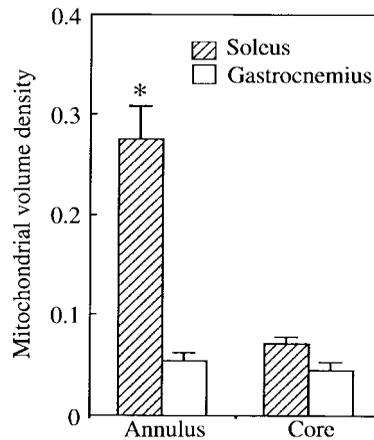


Fig. 7. Mitochondrial volume density in the subsarcolemmal (Annulus) and interfibrillar (Core) areas of slow oxidative fibers of the soleus and fast glycolytic fibers of the gastrocnemius muscles. The columns represent mean values. The bars indicate 1 S.E.M. Figures in parentheses are numbers of observations. \*Significantly different from soleus annulus mitochondrial volume density ( $P < 0.01$ ).

Table 1. Oxidative capacity of the subsarcolemmal and interfibrillar regions of slow oxidative (SO) and fast glycolytic (FG) fibers in the rat

Substrate	Fiber type	Oxidative capacity (nmol O <sub>2</sub> min <sup>-1</sup> ml <sup>-1</sup> tissue)	
		Subsarcolemmal	Interfibrillar
Glutamate/malate	SO	2380	1077
	FG	1072	905
Succinate/rotenone	SO	5910	1460
	FG	1400	1050

however, used the whole gastrocnemius muscle from the rat, a muscle composed of approximately 60% FG and 40% FOG fibers and a very small proportion of SO fibers. These results cannot be directly compared with ours, which were obtained from studies in muscles or areas of muscles with almost pure SO fibers (soleus) and FG fibers (white area of the medial head of the gastrocnemius). It is possible that the lower oxidative capacity of the polytron mitochondria reported by Krieger *et al.* (1980) is the result of the significant proportion of FOG fibers in the muscle they studied. Unfortunately, in the rat, there seems to be no muscle made up of predominantly FOG fibers. We have conducted preliminary studies with the red portion of the vastus lateralis, a muscle with a majority of FOG fibers (Ariano *et al.* 1973) and have found that the oxidative capacity of the polytron mitochondria when using NADH-generating substrates was only 45% of that of the nagarse mitochondria. However, no differences were evident when succinate/rotenone was used as a substrate. This indicates that the oxidative differences between polytron and nagarse mitochondria are limited to oxidative myocytes (SO and FOG fibers) and become evident only when NADH-generating substrates are used.

There is evidence that the isolation technique effectively separates the subsarcolemmal and interfibrillar mitochondrial subpopulations. Palmer *et al.* (1977, 1985) and Krieger *et al.* (1980) have presented microscopic evidence indicating that after a short shock with the polytron tissue processor most of the subsarcolemmal mitochondria are released. The bulk of the interfibrillar mitochondria remains trapped by the fibrils and is released only after treatment with proteolytic enzymes that digest the myofibrils. Nevertheless, it has been argued that the reported differences between isolated polytron and nagarse mitochondria are the result of experimental manipulations during the isolation procedures (Matlib *et al.* 1981). However, Palmer *et al.* (1985) have provided evidence that the difference in oxidative capacity between these two mitochondrial subpopulations cannot easily be attributed to the isolation procedure. Furthermore, the observation that both mitochondrial populations from the soleus oxidize succinate in the presence of rotenone at the same rate provides support for the belief that the differences observed are not the result of the isolation procedure. Additional support for the idea that the differences in oxidative capacity are not the result of the isolation procedure is provided by the observation that the oxidative capacity of the mitochondrial subpopulations from the gastrocnemius show similar oxidative capacities with all the substrates used.

Hoppeler *et al.* (1987) have reported that, in SO fibers from the soleus and FG fibers from the gracilis of the cat, the inner membrane surface density of the mitochondria located underneath the plasma membrane (subsarcolemmal mitochondria) is similar to that of the mitochondria located between the myofibrils (interfibrillar mitochondria). This seems also to be the case for the soleus and the gastrocnemius of the rat. Cytochromes  $c+c_1$  and  $a+a_3$  are markers of the inner mitochondrial membrane and we found similar concentrations of cytochromes in the polytron and nagarse mitochondria in the two muscles (Figs 4 and 5). Because of the link between electron transport, oxygen consumption and the molecular structures of the inner mitochondrial membranes, a correlation is likely to exist between oxidative capacity and the cytochrome content of the mitochondrial membranes (Schwerzmann *et al.* 1989) and, therefore, it should be expected that polytron and nagarse mitochondria show the same oxidative capacity. This was the case for the mitochondrial subpopulations isolated from the gastrocnemius. However, our results in the soleus and those of Krieger *et al.* (1980) in muscles of the hind limb show that polytron mitochondria have a lower capacity to oxidize NADH-linked substrates than do nagarse mitochondria. These discrepancies can be explained if we consider that the rate at which the respiratory chain works is also affected by the rate at which reducing equivalents are made available to it. This situation might not be the same for the polytron mitochondria of the soleus and the other mitochondrial subpopulations. The activity of citrate synthase, an important rate-controlling enzyme of the Krebs cycle, was lower in the polytron mitochondria of the soleus than in the other mitochondrial subpopulations (Fig. 6) and this might result in a lower rate of reducing equivalents being made available to the electron transport chain of the polytron mitochondria of the soleus.

The physiological significance of mitochondria with different oxidative capacities in some fiber types is not clear. Some insight can be obtained if the oxidative capacities of the isolated mitochondrial subpopulations are combined with the distribution of

mitochondrial volume density in different fiber types. The products of these mitochondrial volume densities and the oxidative capacities of the mitochondrial subpopulations were calculated. It was assumed that mitochondrial protein constitutes 25 % of mitochondrial weight and that the density of mitochondria and muscle is 1.00. The results of these calculations (Table 1) show that, in the soleus fibers, the oxidative capacity per unit tissue volume in areas next to the sarcolemma is twice as great as that in the core of the myocyte. It is also evident from these calculations that there is no difference between the oxidative capacity in the two areas of the glycolytic fibers. Furthermore, their oxidative capacity is similar to that of the core of the slow oxidative fibers. It is evident that the much higher oxidative capacity in the area next to the sarcolemma in the oxidative fibers is the result of a fourfold greater mitochondrial volume density. This difference becomes even more marked if similar calculations are carried out using the oxidative capacity values obtained when using succinate plus rotenone as substrate (Table 1).

Mitochondrial volume density and oxidative capacity are directly correlated with O<sub>2</sub> consumption and energy demands in different animal tissues (Weibel, 1979*b*). A similar relationship is likely to exist within the cell. Cellular areas with higher energy demands should have a greater oxidative potential than areas with lower energy demands. Thus, the subsarcolemmal region of the SO fibers must have higher sustained energy demands than the core of the SO fibers and the subsarcolemmal and core areas of the FG fibers. Our results also indicate that the much greater oxidative capacity of a muscle made up largely of SO fibers, when compared with one made up largely of FG fibers, appears to be due to the much higher mitochondrial volume density, especially in areas close to the membrane.

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