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The Turnover of Cytochrome *c* in Different Skeletal-Muscle Fibre Types of the Rat

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The turnover of cytochrome *c* was determined in the three skeletal-muscle fibre types of adult male rats by a kinetic analysis that followed the time course of cytochrome *c* content change. Confirming evidence was obtained with double-labelling studies using δ-aminolaevulinate. Cytochrome *c* turnover was most rapid in the low-oxidative fast-twitch white fibre [$t_{\frac{1}{2}}$ (half-life) about 4 days], slowest in the high-oxidative fast-twitch red fibre ($t_{\frac{1}{2}}$ 9–10 days) and relatively rapid in the high-oxidative slow-twitch red fibre ($t_{\frac{1}{2}}$ 5–6 days). Thus cytochrome *c* turnover does not strictly conform to either the appearance (i.e. red or white) or the contractile characteristics (i.e. fast or slow) of the muscle fibres. The synthesis rates needed to maintain the corresponding cytochrome *c* concentrations, however, were similarly high in the two mitochondria-rich red fibre types. These data illustrate that both the synthesis and degradation processes are important in establishing the cytochrome *c* concentrations that distinguish the different skeletal-muscle fibre types.

Mammalian skeletal muscle is composed of different muscle fibre types that are distinguished on the basis of physiological and biochemical factors (Close, 1972). A functional difference in contractile response is readily apparent between the fast and relatively slow contracting fibres (Burke *et al.*, 1971). This is probably related to the differences in myosin subunits (Sarkar *et al.*, 1971) and myosin adenosine triphosphatase specific activity (Bárány, 1967). In many mammals the fast-contracting fibres can be further subdivided according to their oxidative capacity (Baldwin *et al.*, 1972; Peter *et al.*, 1972). In rats, for example, there is a fast-twitch red fibre which has the highest oxidative capacity, a fast-twitch white fibre with the lowest oxidative capacity, as well as the slow-twitch red fibre, which has an intermediate oxidative capacity. These differences in oxidative capacity are apparent from a variety of mitochondrial markers of oxidative metabolism. This includes the capacity for pyruvate oxidation, non-esterified fatty acid oxidation, the enzymes of the tricarboxylic acid cycle, cristae components and other processes characteristic of mitochondrial function (see Holloszy & Booth, 1976). These differences in mitochondrial capacity are probably instrumental in supporting the unique performance characteristics of the red fibre types during repeated contractions.

The greatest difference in oxidative capacity is found between the fast-twitch red and the fast-twitch white fibres. For example, the cytochrome *c* concentration in the fast-twitch red fibre is approx. 4 times that of the fast-twitch white fibre (Winder *et al.*, 1974; Terjung & Koerner, 1976). This greatly increased cytochrome *c* concentration could be established by a greater synthesis of cytochrome *c*, by a decrease in the degradation rate of cytochrome *c*, or a combination of both factors. The present study was designed to provide a quantitative assessment of the cytochrome *c* degradation and synthesis rates that distinguish the fibre types.

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Experimental

The turnover of cytochrome *c* was determined by following the time course of concentration change during the transition from one steady-state content to another as described by Schimke & Doyle (1970). Four separate conditions were used to alter the steady-state concentration of cytochrome *c*. The deviation away from normal values was established by a hypothyroid state and by exercise training. A hypothyroid condition produces a general decline in muscle cytochrome *c* concentration to about one-half normal (Terjung & Koerner, 1976). Endurance-type training, such as prolonged treadmill running, causes an increase in oxidative capacity of the working muscles (Holloszy, 1967). This increase in oxidative capacity, as reflected in cytochrome *c* concentration, is dependent on the duration (Fitts *et al.*, 1975) and intensity of exercise (Terjung, 1976), and is most evident in the high-oxidative fibres involved at moderate work loads (Terjung, 1976; Terjung & Koerner, 1976). The return to normal concentrations

of cytochrome *c* was followed after removal of the hypothyroid status and after cessation of exercise training.

The data were analysed by a first-order kinetic model (Schimke & Doyle, 1970) by using a non-linear least-squares procedure. The half-life ($t_{\frac{1}{2}}$) was calculated from the relationship $t_{\frac{1}{2}} = \ln 2/k$, where the derived slope (k) is the degradation rate constant. Thus the half-life for each curve was taken to represent the degradation process for each treatment. The zero-order synthesis rate (S) for each steady-state condition was then calculated from the equation (Schimke & Doyle, 1970) $S = k$[cytochrome *c*].

In addition to the assessment of the degradation rate constants from the transition studies, the relative turnovers of cytochrome *c* in the different skeletal-muscle fibre types were evaluated by the double-isotope technique introduced by Schimke (1970). In this procedure two injections of a precursor, one labelled with ^3H and the other with ^{14}C, are administered to the animals. A time interval is allowed between injections to permit the loss of the label that was initially incorporated according to the degradation rate apparent in that tissue. The animals are killed after the second injection. A comparison of the radioactivity from the second injection with that remaining from the first injection should reflect the relative turnover of the compound, since the relative quantity of the first injection that remains is dependent on its turnover rate. The reference to the second injection permits a relative comparison with that amount originally incorporated but not subjected to any significant degree of degradation. This procedure assumes that the conditions for incorporation within a given tissue are similar during each label administration. In this study labelled δ-aminolaevulinate was used, since it is specifically incorporated into the haem portion of cytochrome *c* (Druyan *et al.*, 1969). Since the haem and apoprotein portions of cytochrome *c* are covalently bound and probably turn over as a unit (Druyan *et al.*, 1969), these data should complement the transition data where the entire cytochrome *c* molecule was isolated.

Animal care and experimental protocol

Adult Wistar rats (Carworth Laboratory, Portage, MI, U.S.A.), weighing between 300 and 400g, were housed individually with a 12h light–dark cycle and provided with food and water *ad libitum*. The animals involved in the training programme were exercised on a motor-driven treadmill (Quinton model 42–15) by a protocol used previously (Terjung, 1976). This required a daily exercise bout up a 15% grade at 26.8m/min first for 15min/day and increasing by 3min/day until running for 1h/day. Animals that were detrained were first exercised for 14 weeks to ensure an elevated steady-state concentration of cytochrome *c*. The hypothyroid status was caused by thyroidectomy (rats supplied by Hormone Assay Laboratory, Chicago, IL, U.S.A.) or by daily injections of propylthiouracil (40mg/kg subcutaneous). The thoroughness of the surgical procedure by this supplier was verified previously (Tipton *et al.*, 1968; Terjung & Koerner, 1976). The daily dose of propylthiouracil used in the present studies was above that necessary to eliminate completely thyroidal thyroxine and tri-iodothyronine production (Iino *et al.*, 1961). The removal of the hypothyroid condition was established by cessation of the daily propylthiouracil injections. The return of thyroid-hormone influence was not immediate, since the cytochrome *c* concentrations remained uniformly depressed for about 1 week. This was most probably due to the delayed clearance of propylthiouracil. The cytochrome *c* content then began an abrupt return toward normal and followed the transition shown in the Figures.

Groups of four animals each were taken at various times after the initiation of the treatment. Immediately after decapitation, the hind-limb muscles were exposed and sectioned for cytochrome *c* extraction. Tissue sections of predominantly fast-twitch red, fast-twitch white and slow-twitch red fibres were obtained from the red vastus, white vastus and soleus muscles respectively (Baldwin *et al.*, 1972). The whole gastrocnemius muscle was used as representative of a mixed-fibre-type skeletal muscle (Ariano *et al.*, 1973).

In the double-isotope experiment, six rats were injected (intraperitoneal) with 54.2 μCi of δ-amino-[3,5-^3H]laevulinate (5Ci/mmol)/100g body wt. (New England Nuclear, Boston, MA, U.S.A.). Then 12 days later these animals were given 20.0 μCi of δ-amino[4-^{14}C]laevulinate (55.1mCi/mmol)/100g body wt. and killed the following day. Tissues were sectioned as described above. Cytochrome *c* was extracted from the varous tissues and purified as described below. Incorporated radioactivity was determined by standard liquid-scintillation procedures (Anderson & McClure, 1973) at efficiencies of 30% and 64% for ^3H and ^{14}C respectively.

Cytochrome c assay

Cytochrome *c* was extracted and purified by a procedure used previously (Terjung, 1976). Briefly, this involved an initial extraction of the muscle with 175mM-KCl/10mM-Tris/HCl/2mM-EDTA, pH7.4 (1g of tissue/20ml volume), then a re-extraction of the 30000*g*-10min pellet with the initial volume of 20mM-potassium phosphate, pH7.4, all at 4°C. The 30000*g* supernatants were combined and cytochrome *c* was isolated from an Amberlite CG-50 column (Terjung, 1976). The cytochrome *c* isolated by this procedure is free from other haem proteins and yielded an oxidized-minus-reduced absorbance spectrum that was identical with that for purified cytochrome *c* (Margoliash & Frohwirt, 1959). Cyto-

chrome *c* recovery by this extraction and purification procedure is 80% (Terjung, 1976).

Results

Turnover of cytochrome c in the different skeletal-muscle fibre types

Our estimates of turnover are based on the time course of cytochrome *c* concentration changes (Fig. 1). This procedure assumes that, after initiation of the treatment, the synthesis and degradation rate constants remain unchanged throughout the transition period (Schimke & Doyle, 1970). However, since each treatment ultimately establishes a new cytochrome *c* concentration, there obviously must be an initial shift in the synthesis and/or the degradation rate constant. Thus there is the added expectation that any initial change in these processes must occur in a manner that is rapid relative to the half-life (Schimke & Doyle, 1970). A significant error could be introduced if this were not the case, especially if the transition is not followed to completion. It can be shown, however, that the error would be most evident during the initial stages of the transition and cause a non-linear log response. Thus a high correlation would be expected for the log plot established over a number of half-lives, if the synthesis and degradation rate constants were uniform throughout the transition. This expectation seems to be substantially fulfilled with the transitions used in the present study. High correlation coefficients (r), between −0.95 and −0.99, were obtained for all of the transitions except the training curve for red vastus ($r = -0.82$), and the curve for white vastus muscle in post-hypothyroid rats ($r = -0.71$). Representative first-order curves are shown in Fig. 2.

The degradation rate constant apparent during a transition is expected to reflect the cellular conditions for turnover established by the treatment under consideration. The synthesis rate of cytochrome *c*, on the other hand, exerts its influence on the ultimate concentration of cytochrome *c* achieved at the end of the transition period. The two treatments used in our studies where the cytochrome *c* concentration returned to normal [i.e. cessation of training (C curves) and the removal of the hypothyroid state (D curves)] should then be most representative of the turnover of cytochrome *c* in normal rat muscle. In addition, however, we evaluated the transitions caused by two other procedures, namely exercise training (A curves) and hypothyroidism (B curves). Since these procedures altered the cytochrome *c* concentrations away from normal, the transitions do not necessarily represent the cellular control of normal muscle. This is especially true if these treatments significantly alter the degradation rate constant of cytochrome *c* from normal. On the other hand, if their primary influence is to change the synthesis rate of cytochrome *c*, then the time course throughout the transition would be

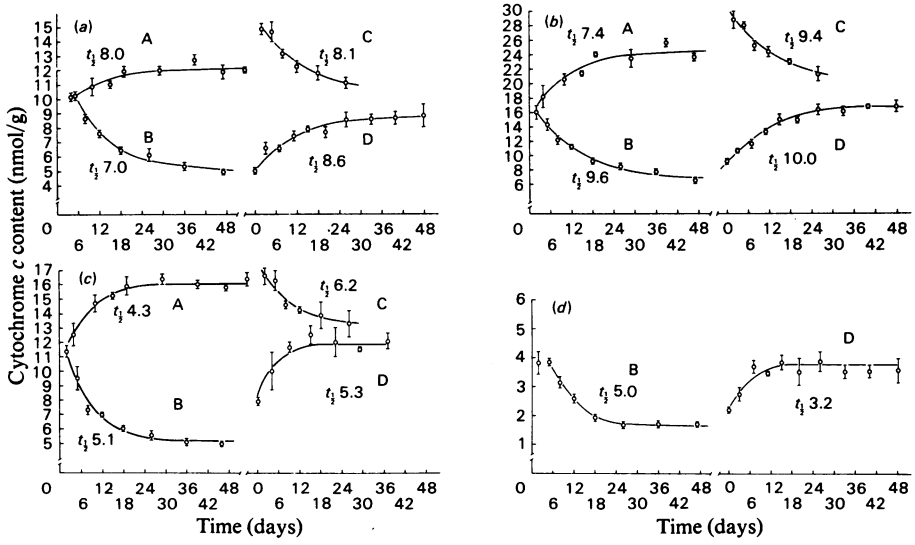

Fig. 1. *Transition curves of the change in cytochrome c concentration as a function of time for the gastrocnemius (a, a mixed muscle), red vastus (b, fast-twitch red), white vastus (d, fast-twitch white) and soleus (c, slow-twitch red) muscle sections*
Cytochrome *c* content (mean ± S.E.M. shown) was altered away from normal by exercise training (A curves) and by a hypothyroid status (B curves), and returned to normal by cessation of training (C curves) and by removal of the hypothyroid status (D curves) (see the Experimental section). Each half-life ($t_{\frac{1}{2}}$, in days) was calculated from the degradation rate constant obtained from the slope of the first-order curve fit.

similar to that of normal muscle. Thus each specific treatment is potentially useful in discriminating between the degradation rate constants of each specific muscle fibre type. However, the training programme used in this study was not sufficiently intense to cause a large enough increase in cytochrome *c* in the white vastus fibres to permit the transitions to be accurately followed.

The turnover of cytochrome was most rapid in the white vastus and slowest in the red vastus muscle fibres (Fig. 1). The slow red-type fibre of the soleus muscle exhibited a turnover that was nearly as rapid as in the fast-twitch white fibre. This appeared to be true for each of the specific treatments used. The turnover of cytochrome *c* in the gastrocnemius was between that of the fast-red and fast-white fibres, as expected from the composition of this mixed muscle (Ariano *et al.*, 1973). On the basis of the contribution of each fibre type to the total cytochrome *c* content in this mixed muscle, the fast-twitch red fibre is most important quantitatively (about 65%), with the fast-twitch white and slow-twitch red fibres less so, approx. 30 and 5% respectively. Thus the turnover of cytochrome *c* in the gastrocnemius ($t_{\frac{1}{2}} = 8$ days) should be most similar to that of the fast-twitch red fibre ($t_{\frac{1}{2}} = 9$–10 days).

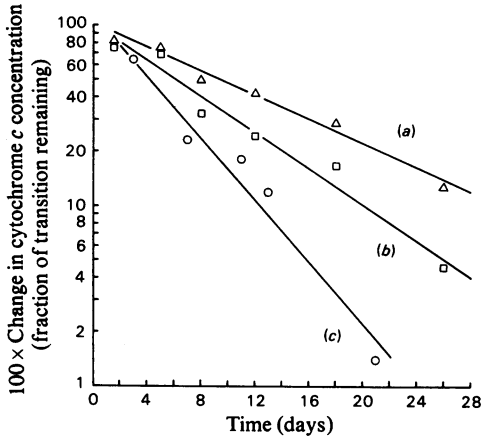

Fig. 2. *First-order transition curves of the change in cytochrome c concentration as a function of time representative of normal red vastus* (*a*, *fast-twitch red*, $r = -0.99$), *soleus* (*b*, *slow-twitch red*, $r = -0.98$) *and white vastus* (*c*, *fast-twitch white*, $r = -0.98$) *muscles*

Each curve is calculated from the data established by conditions that returned the cytochrome *c* concentration to normal (see the Experimental section). An exception is the white vastus (*c*), which also includes the transition data caused by the hypothyroid condition.

Overall estimates of the half-lives and corresponding degradation rate constants for each muscle fibre are summarized in Table 1. The synthesis rates necessary to maintain the cytochrome *c* concentrations of normal muscle in the face of the corresponding degradation rates have also been calculated. These results indicate that even though the turnover of cytochrome *c* is the most rapid in the fast-twitch white fibres, the relatively low concentration of cytochrome *c* can be maintained by a relatively low synthesis rate. In contrast, the relatively high synthesis rate in the fast-twitch red fibre is necessary to maintain the high cytochrome *c* concentration, even though the turnover is relatively slow. Thus the higher concentration of cytochrome *c*, approx. 4 times that of the fast-twitch white fibres, is due to nearly equal influences of an elevated synthesis and a diminished degradation rate constant. The synthesis rate of the slow red fibre of the soleus muscle is relatively high, since both the turnover and the concentration of cytochrome *c* are fairly high. Thus the greater content of cytochrome *c* in the slow-twitch red fibre compared with the fast-twitch white fibre is due primarily to the increased synthesis rate.

Double-label technique with δ-aminolaevulinate

The assessment of the relative degradation rates by the double-isotope technique provides a separate means to evaluate cytochrome *c* turnover. This procedure, however, is subject to error, owing to

Table 1. *Summary of cytochrome c metabolism in the different skeletal-muscle fibre types in the rat*

The degradation rate constants (*k*) are the slopes of the first-order curves (see Fig. 2) representative of the values obtained from the transition studies. The cytochrome *c* concentrations represent the average values of 40 or more normal animals. The synthesis rate (*S*) is that production needed to maintain the cytochrome *c* concentration of each fibre type in the face of the corresponding degradation rate.

	$t_{\frac{1}{2}}$ (days)	Degradation rate constant, *k* (per day)	Cytochrome *c* (nmol/g)	Synthesis rate, *S* (nmol/day per g)
Gastrocnemius (mixed)	8	0.087	9.37	0.82
Red vastus (fast-twitch red)	9–10	0.073	16.85	1.23
White vastus (slow-twitch white)	4	0.173	4.16	0.72
Soleus (slow-twitch red)	5–6	0.126	11.08	1.40

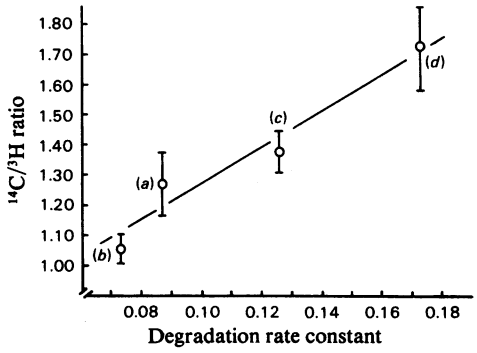

Fig. 3. *Relationship between the cytochrome c degradation rate constants (k) and the $^{14}C/^{3}H$ ratios for the different skeletal-muscle fibre types of the rat*

The degradation rate constants were obtained from the transition studies (see Table 1) and the $^{14}C/^{3}H$ ratios (means±S.E.M.) were obtained by the double-isotope technique by using δ-aminolaevulinate (see the Experimental section). (*a*) Gastrocnemius; (*b*) red vastus; (*c*) soleus; (*d*) white vastus.

isotope reutilization. δ-Aminolaevulinate, which is specifically incorporated into the haem portion of the cytochromes and eliminated as bilirubin, provides a nearly 'ideal' tracer for liver (Aschenbrenner *et al.*, 1970). However, this does not appear to be true for all tissues, since this precursor is subject to reutilization in skeletal muscle (Terjung, 1975; Booth & Holloszy, 1977). As a result, radioisotopic-decay curves of skeletal-muscle cytochrome *c* underestimate the degradation rate constant (Terjung *et al.*, 1973). Nonetheless, the fractional error introduced appears to be similar for all of the muscle fibre types (R. L. Terjung, unpublished work). Thus the double-label technique should still be useful in distinguishing differences in the turnover of cytochrome *c* in the different types of skeletal muscle. Although our schedule of radioisotope administration did not permit optimal separation of the $^{14}C/^{3}H$ ratios (Zak *et al.*, 1977), it did provide a sufficient resolution to meaningfully reflect differences in cytochrome *c* degradation in the fibre types. Therefore there should be a high correlation between the double-label ratios and the degradation rate constants obtained from the transition studies. This relationship is shown in Fig. 3. The high correlation ($r = 0.97$) reinforces the transition data, which show that cytochrome *c* turnover is most rapid in the fast-twitch white fibre (white vastus) and slowest in the fast-twitch red fibre (red vastus).

Discussion

The significant reutilization of amino acids in skeletal muscle (Waterlow & Stephen, 1968) imparts a limitation on the use of labelled precursors to follow the degradation rate of muscle proteins by the loss of radioactivity. Even the use of a specialized precursor such as δ-aminolaevulinate, which provides a nearly ideal tracer for haem proteins in other tissues (Druyan *et al.*, 1969), is not appropriate for skeletal muscle (Terjung, 1975; Booth & Holloszy, 1977). Thus we were attracted to a kinetic analysis for determining the turnover of cytochrome *c* in the different skeletal-muscle fibre types. This method has been used on numerous occasions to study the turnover of cellular enzymes, including mitochondrial components (cf. Schimke, 1973). Although this procedure requires certain assumptions, they seem to be reasonably well fulfilled in our experiments (see the Results section). The use of a mitochondrial component, such as cytochrome *c*, to describe the transition from one steady state to another was considered desirable, since this determination is quantitative and would not be subject to alterations in specific activity, as could be the case with the use of an enzyme marker. In addition, it is probable that the changes in cytochrome *c* represent changes in a major portion of the mitochondrial cristae, since there seems to be a co-ordinated change in the cristae components whenever changes in mitochondrial contents are produced (Holloszy, 1967; Winder & Holloszy, 1977). More importantly, the turnover of several cristae components is similar. Cytochrome *c*, cytochrome *b* and haem *a* exhibit a uniform turnover in rat heart and liver (Druyan *et al.*, 1969; Aschenbrenner *et al.*, 1970). Furthermore, these turnover rates are fairly similar to the turnover of the total mitochondrial protein fraction from the same tissues (Aschenbrenner *et al.*, 1970). Thus our results for cytochrome *c* probably represent a significant portion of the mitochondrial inner membrane. However, it is clear that the handling of cytochrome *c* cannot represent the entire mitochondrion, since there is a considerable heterogeneity in the turnover of many mitochondrial components (see Schimke, 1970).

One distinctive feature that characterizes each of the three skeletal-muscle fibre types of the rat is their mitochondrial content. This is illustrated by the cytochrome *c* concentration, which varies over an approx. 4-fold range (Table 1). Our turnover data indicate that the different skeletal-muscle fibre types establish their respective concentrations of cytochrome *c* by quantitatively different means. For example, the relatively high-oxidative fibres (i.e. the fast-twitch red and the slow-twitch red) exhibit a cytochrome *c* synthesis rate approximately twice that of the low-oxidative fast-twitch white fibre. Thus, as might be expected, the higher concentrations of this cytochrome are in large measure due to its increased production. This difference in production, however, is not the sole factor that accounts for differences in cytochrome *c* content. The turnover of cytochrome *c* was found to differ considerably in the fast-contracting fibres. The degradation rate constant was approxi-

mately twice as much in the white vastus as in the red vastus muscle section. Thus the approx. 4-fold greater concentration of cytochrome *c* in the red vastus is due to nearly equal influences of a greater synthesis and a decreased degradation of cytochrome *c*. In contrast with the situation in the fast-twitch red fibre, cytochrome *c* turnover in the slow-twitch red fibre is relatively rapid. Thus the major factor establishing the difference in cytochrome *c* concentrations between these relatively high-oxidative fibres is a quantitative difference in the degradation process (Table 1). On the other hand, the production of cytochrome *c* seems to be the major factor responsible for the distinction in cytochrome *c* content between the slow-twitch red fibre and the fast-twitch white fibre. In this context, it is noteworthy that the differences in neither cytochrome synthesis nor degradation rates in the different skeletal-muscle fibre types correspond to the widely used classifications of muscle based on either contractile characteristics (i.e. fast or slow) or oxidative capacity (i.e. red or white). Although the cytochrome *c* synthesis rate is, in general, correlated with oxidative capacity of the muscle fibres, the degradation rate constant is not (Table 1). Furthermore, the cytochrome *c* degradation rate constant for each fibre type does not seem to correspond to the fibre's contractile properties. This illustrates the need to consider each of the fibre types, and not simply its appearance or contractile response, when selecting a muscle for study. This is especially evident when one considers the many inherent differences between the fibre types, as seen, for example, in the pathways for energy metabolism (Baldwin *et al.*, 1972, 1973; Pette *et al.*, 1973).

This involvement of both synthesis and degradation to establish differences in cytochrome *c* content is not surprising. Changes in these processes have been shown to account for the altered cytochrome *c* concentrations caused by abnormal thyroid status (Gross, 1971; Booth & Holloszy, 1975). These influences on cytochrome *c* metabolism, however, do not significantly change the relative differences in cytochrome *c* content between the fibre types (Baldwin *et al.*, 1972; Terjung & Koerner, 1976). Thus more information is needed to account for the differences in cytochrome *c* metabolism evident in the three skeletal-muscle-fibre types of the rat. This information may be fundamental to an understanding of the developmental (Baldwin *et al.*, 1978) and neurotrophic (Pette *et al.*, 1973) influences that affect muscle fibre differentiation and oxidative capacity.

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