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Alteration in Skeletal Muscle Mitochondria of Cold-Acclimated Rats: Association with Enhanced Metabolic Response to Noradrenaline

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Abstract

This paper reports a search for structural changes in skeletal muscle mitochondria of cold-acclimated rats. Histochemical studies (succinic dehydrogenase) show that there appears to be a higher proportion of red fibers in the semitendinosus muscle of the cold-acclimated rat and that the white region of this muscle contains fibers which resemble intermediate fibers. Electron micrographs show an apparently larger number of small mitochondria in both red and white fibers. Counts of mitochondria isolated from skeletal muscle show that there are more mitochondria per gram of both red and white muscle in the cold-acclimated rat than in the non-acclimated control rat. Each mitochondrion contains less protein and less cytochrome oxidase. Thus the mitochondrial mass per gram of red and white muscle is not altered, as indicated by the unchanged content of mitochondrial protein and of cytochrome oxidase per gram of muscle. Thus there appears to be a repackaging of mitochondrial material into smaller units in the skeletal muscle of the cold-acclimated rat. The alteration is shown to be associated with the adaptive state of the rat. No change occurs in muscle mitochondria of cold-acclimated rats in which the development of the enhanced metabolic response to noradrenaline, a measure of the extent of adaptation, is inhibited by treatment with oxytetracycline. The change in skeletal muscle mitochondria disappears when the enhanced metabolic response to noradrenaline in rats which are already cold-climated is reversed by treating the rats with oxytetracycline while they continue to live in the cold. The change in

muscle mitochondria also disappears when the cold-acclimated rat undergoes deacclimation after return to room temperature. The alteration in muscle mitochondria is thus not associated either with shivering or with a high metabolic rate. Skeletal muscle of the cold-acclimated rat is known to be an important site of heat production in the course of nonshivering thermogenesis; that is, it can undergo a considerable increase in metabolic rate in the absence of shivering on exposure of the cold-acclimated rat to cold. The metabolic basis of nonshivering thermogenesis is in an enhanced capacity of the tissues of the cold-acclimated rat, principally skeletal muscle, to respond by an increase in metabolic rate to the large amounts of noradrenaline secreted by the nerve endings of the sympathetic nervous system as a consequence of cold-exposure. The mechanism by which the metabolic response to noradrenaline in the cold-acclimated rat can be enhanced is unknown. The structural alteration observed in the skeletal muscle mitochondria of the cold-acclimated rat may indicate a functional alteration responsible for the enhanced capacity of the muscle to respond to noradrenaline by an increase in metabolic rate.

Introduction

When a rat is placed in the cold (4°) it shivers in order to increase its heat production and maintain its body temperature. After having lived in the cold for 3 to 4 weeks the rat no longer shivers; it is then said to be cold-acclimated [1, 2]. Nevertheless, the metabolic rate of the cold-acclimated rat is as high as was that of the shivering rat. The term nonshivering thermogenesis is used to describe the increased heat production which occurs in the cold-acclimated rat living in the cold. Nonshivering thermogenesis is believed to represent a greatly enhanced calorigenic response of the rat to the large amounts of noradrenaline secreted by its sympathetic nerve endings, activated by the exposure to cold (see [3] for review). The capacity of the rat to respond to noradrenaline by raising its metabolic rate increases during the period of cold-acclimation [4].

The site of nonshivering thermogenesis and of the enhanced calorigenic response to noradrenaline appears to be principally skeletal muscle and brown adipose tissue. A major contribution by the liver and other viscera would appear to be excluded by the experiments of Depocas [4, 5] in which evisceration was shown not to alter cold-induced nonshivering thermogenesis [5] or the enhanced metabolic response to noradrenaline [4] of cold-acclimated rats. A small and unknown contribution to nonshivering thermogenesis probably occurs in liver [6, 7]. Brown adipose tissue is undoubtedly a site of nonshivering thermogenesis [6, 8] but its quantitative contribution is rather small [9, 10]. The only major organ remaining in consideration as a site of nonshivering thermogenesis is, by exclusion, thermogenesis and a metabolic response to noradrenaline can occur in skeletal muscle [11, 12]. The quantitative contribution of skeletal

muscle to total nonshivering thermogenesis has not been measured; however, the increase in muscle is large (280%) compared with the increase in the intact rat (70%) [12]. On the basis of total metabolic capacity, as measured by cytochrome oxidase content and by blood flow, it is possible to calculate that up to 70% of nonshivering thermogenesis must occur in skeletal muscle [6].

The mechanism of nonshivering thermogenesis and of the enhanced calorigenic response to noradrenaline is not well understood. The increased calorigenic response to noradrenaline in the cold-acclimated rat could conceivably be mediated by an increase in the amount of the noradrenaline-sensitive adenyl cyclase system in the target tissues. However, no change in the amount of basal, noradrenaline-stimulated or fluoride-stimulated adenyl cyclase has been found in skeletal muscle of cold-acclimated rats [13]. No mechanism is known for nonshivering thermogenesis in skeletal muscle and the nature of the adaptive alteration in skeletal muscle which permits the enhanced metabolic response to noradrenaline is not understood. Mitochondria isolated from skeletal muscle of cold-acclimated rats have normal ADP/O, Ca^{2+}/O, and respiratory control ratios [14].

A clue to the possible nature of this alteration in skeletal muscle of cold-acclimated rats was obtained in experiments in which the half-lives of mitochondrial proteins of various organs of the cold-acclimated rats were measured [15]. A decrease in half-life in two out of the five groups of mitochondrial proteins studied ("structural proteins" and "contractile proteins") was observed in skeletal muscle and brown adipose tissue, tissues in which nonshivering thermogenesis occurs; no difference in half-life of any group of proteins was observed in mitochondria of liver or kidney, tissues in which nonshivering thermogenesis does not occur. Thus, by virtue of its location this decrease in half-life of some mitochondrial proteins is associated with the capacity for nonshivering thermogenesis. The decrease suggests that mitochondrial protein metabolism is altered in cold-acclimated rats.

That mitochondrial protein synthesis is necessary for cold-acclimation to occur is shown by the inhibition by oxytetracycline, an inhibitor of mitochondrial protein synthesis, of the development of the enhanced response to noradrenaline in rats living in the cold [16]. These rats have a high metabolic rate while living in the cold and are presumably shivering. The inhibition was associated with prevention of the usual mitochondrial proliferation in brown adipose tissue during cold-acclimation [16] and with inhibition of mitochondrial protein synthesis in muscle [17]. These results suggest that accelerated mitochondrial protein synthesis might occur in skeletal muscle and brown adipose tissue in association with the development of the cold-acclimated state.

This paper describes the search for an alteration in skeletal muscle mitochondria in cold-acclimated rats. The assumption that there should be an alteration is based upon the lines of evidence described above, i.e., that muscle is a major site of nonshivering thermogenesis and of enhanced metabolic response to noradrenaline, that the receptor system for noradrenaline (the adenyl cyclase system) of the skeletal muscle of cold-acclimated rats is unchanged and that altered mitochondrial protein synthesis occurs in skeletal muscle of cold-acclimated rats and might be expected to be indicative of an altered form and/or function of the mitochondria.

The muscle chosen for study was the semitendinosus. There are two reasons for this choice. Firstly, the ultrastructure and fiber distribution of this muscle have been extensively documented by Gauthier [18, 19, 20]. Secondly, it was necessary to be able to distinguish between the different fiber types of the muscle; this is not difficult for the semitendinosus because the red (consisting primarily of red and intermediate fibers) and white (consisting primarily of white fibers) portions of this muscle can be readily seen and separated, unlike most muscles of the rat in which there is random distribution of fibers.

Materials and Methods

Animals

Male Holtzman rats lived at 4°C (cold-acclimated) or at 26°–28° (control) in individual open wire mesh cages with lighting from 6 a.m. to 6 p.m. daily. They were killed by decapitation. In the structural studies the rats had lived at their temperature of acclimation for 4 to 5 weeks; their body weights at this time were 327 ± 9.9 g (control) and 244.7 ± 8.5 g (cold-acclimated). For the initial studies in which mitochondria were isolated the rats had lived at their temperature of acclimation for 4 months: mean body weights at this time were 560 ± 4.9 g (controls) and 404.9 ± 5.6 g (cold-acclimated rats). Some rats were injected intramuscularly twice daily with either saline or oxytetracycline (Terramycin, Pfizer; 200 or 300 mg/kg per day). A total of 32 rats were divided into two groups to be kept in the cold (140.0 ± 2.7 g) or at room temperature (136.8 ± 2.4 g). Injections were started while the rats were at room temperature. The next day they were either transferred to the cold or remained at room temperature. Another group of 10 rats (127.5 ± 3.5 g) was kept at 4°C for 2 weeks before injections were started: they were then divided into two groups, one of which received daily injections of saline and the other of which received daily injections of oxytetracycline for a further two weeks. For the

experiments in which deacclimation was studied 56 rats (112.8 ± 5.1 g) were placed either at 4°C or at room temperature for 4 weeks, at which time the rats were transferred from 4°C to room temperature. They were studied on the day of removal from the cold or 1 or 2 weeks later.

Metabolic responses of rats to noradrenaline were measured as described previously [21]. The increase in oxygen uptake during the 30 min of intravenous infusion of noradrenaline (0.5 g/100 cm^2 min) is calculated as ml oxygen/100 cm^2 body surface of rat. Body surface is calculated according to the formula: body surface $(cm^2) = K \times$ body weight $(g)^{2/3}$ where $K = 7.5$ [22].

Preparation of Mitochondria

The semitendinosus muscle was removed from both legs and separated into red and white portions. 5 g of muscle (from 7–8 rats) were sliced with a razor blade and then incubated for 30 min at 0°C in 50 ml of medium containing 0.21 M mannitol, 0.07 M sucrose, 0.01 M HEPES buffer pH 7.4 and 0.01 M EDTA (this is subsequently referred to as mannitol medium) plus heparin, 250 units per ml, 1 mM ATP, and protease, 10 mg (Sigma protease type VII, Subtilisin BPN′). The suspension was homogenized in a Duall homogenizer and diluted to 100 ml with mannitol medium plus heparin. The homogenate was filtered through gauze and then centrifuged for 15 min at 2000 rpm ($650 \times g$) in a swinging bucket rotor (HB 4) of a Sorvall RC-2B refrigerated centrifuge. The supernatant was then centrifuged for 15 min at 9000 rpm ($13{,}000 \times g$). The sedimented mitochondria were resuspended in the mannitol medium by homogenization in a smooth glass homogenizer with a teflon pestle, diluted to 50 ml and recentrifuged for 10 min at 7800 rpm ($10{,}000 \times g$). Mitochondria were again resuspended in a small volume (20 ml) and centrifuged at low speed to remove small particles of debris still present; the sediment from this low-speed centrifugation was washed once and the combined supernatant plus washings were recentrifuged at high speed (10 min at 7800 rpm, $10{,}000 \times g$). The sedimented mitochondria were resuspended in a small volume of the mannitol medium containing heparin and 1% albumin (fatty acid free) to a final mitochondrial protein concentration of 2 mg/ml. Protein estimations were done according to the method of Lowry [23] after precipitating the proteins with trichloroacetic acid and redissolving them in sodium hydroxide.

In subsequent experiments mitochondria were prepared from 10 g of mixed leg muscle of individual rats. The procedure was the same except that the incubation volume was 100 ml and 20 mg of protease was added.

Estimation of Cytochrome Oxidase

The homogenates and mitochondrial fractions were homogenized for 1 min with Lubrol (0.3 mg/mg mitochondrial protein for the mitochondrial fractions or 6 mg/g of muscle for the homogenates) and diluted to 0.05 g of muscle/ml (homogenates) or 1 mg mitochondrial protein/ml (mitochondrial fractions). Cytochrome oxidase was measured polarographically at 37°C in a medium containing 77.5 mM potassium phosphate buffer pH 6.6, 0.2 mM cytochrome c and 20 mM sodium ascorbate in a volume of 3 ml. The solution of cytochrome c was prepared as described by Wharton and Tzagoloff [24] and the concentration of total and reduced cytochrome c checked using the extinction coefficients reported by Yonetani [25]. Activity is expressed as μ atoms oxygen consumed/min.

Counting of Mitochondria

The method of Baranowicz [26] was followed. Erythrocytes from the same rat were washed in the mannitol medium containing heparin while the mitochondria were being prepared. They were diluted to a concentration of $6–9 \times 10^9$ cells/ml. A mixture of 1 ml of the erythrocyte suspension and 1 ml of the mitochondrial suspension were thoroughly mixed and finally diluted to a volume of 4 ml. A few smears were prepared. Immediately after drying the smears were observed under high magnification (×1250) without oil immersion. The number of erythrocytes and mitochondria in 12 different fields from at least two different smears were recorded and from the ratio of cells to mitochondria the number of mitochondria in the suspension was calculated.

Histochemical Methods

Muscles were tied to a wooden splint and frozen in liquid nitrogen. The frozen muscle was placed in a cryostat (–20°C) for sectioning. Sections 10 μ thick were cut with a rotary microtome and mounted on glass slides. Succinic dehydrogenase activity was localized using Nitro-blue tetrazolium according to the method of Nachlas et al. [27]. The sections were finally mounted in glycerol-gelatin and photographed.

Electron Microscopy

Samples of red and white portions of the semitendinosus muscle were prefixed in ice-cold 4% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 for 2 hr. The small rods of muscle (1 mm diameter × 1.5 mm length) were

rinsed three times with 0.1 M phosphate buffer pH 7.4 and fixed for 1 hr in 2% osmium tetroxide in 0.1 M phosphate buffer pH 7.4. The rods were then rinsed in 30% ethanol, dehydrated by successive passages (7 min each) through increasing concentrations of ethanol and embedded in Araldite resin according to the method of Luft [28]. Thin sections (silver-gold) were cut on a Reichert ultramicrotome using glass knives, then stained with lead nitrate and uranyl acetate [29] and examined in an electron microscope at 7000 × magnification.

Results

The semitendinosus of control rats showed the characteristic distribution [18, 19] of fibers rich in mitochondria in the red portion and fibers poor in mitochondria in the white portion (Fig. 1A). The red portion occupied approximately one-third of the total cross-section. In the semitendinosus muscle of the cold-acclimated rat the red portion occupied more than half the total cross-section and the white fibers were more deeply stained (Fig. 1B). There was also an apparent decrease in the size of the fibers, as previously reported by Héroux [30], but the total number remained unchanged.

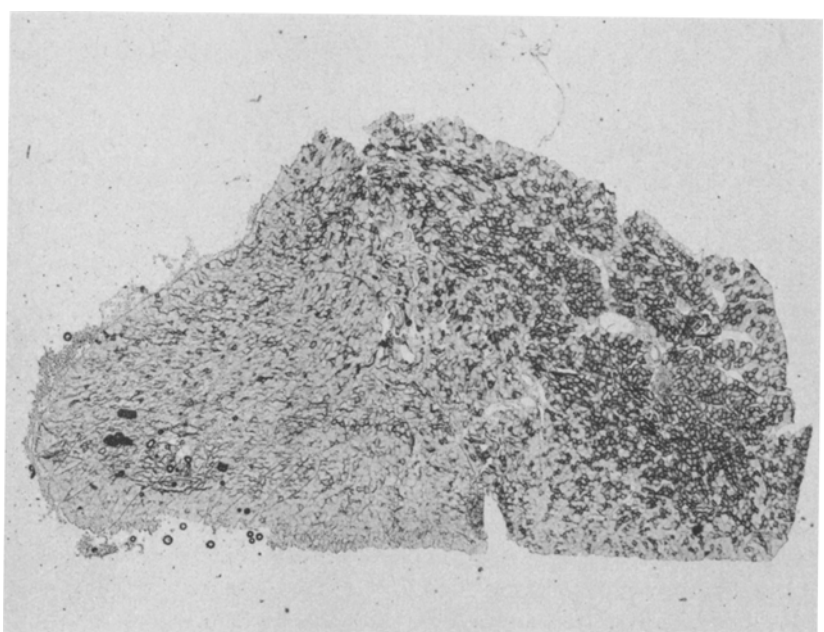


Figure 1a. Cross-section of the semitendinosus muscle of a warm-acclimated rat. Stained for succinic dehydrogenase × 15.

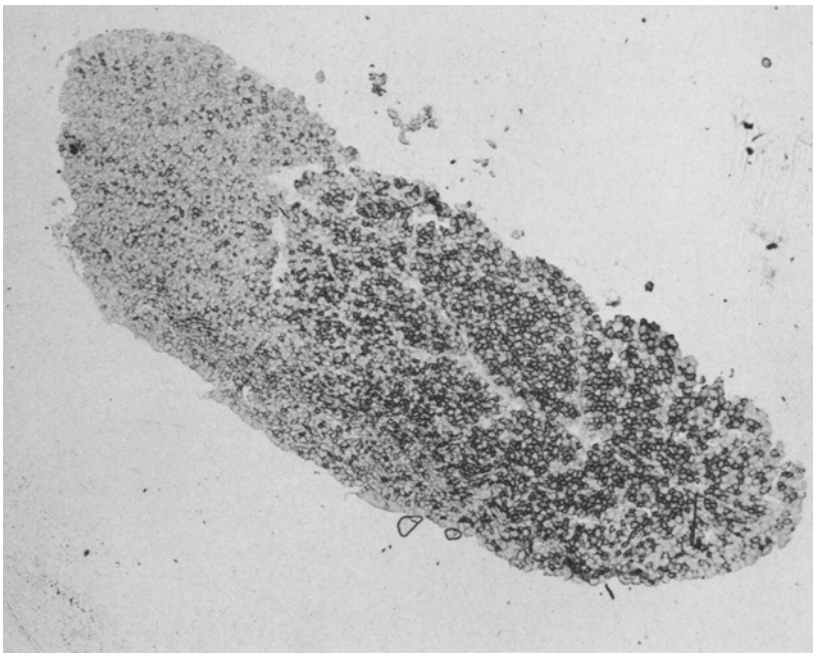
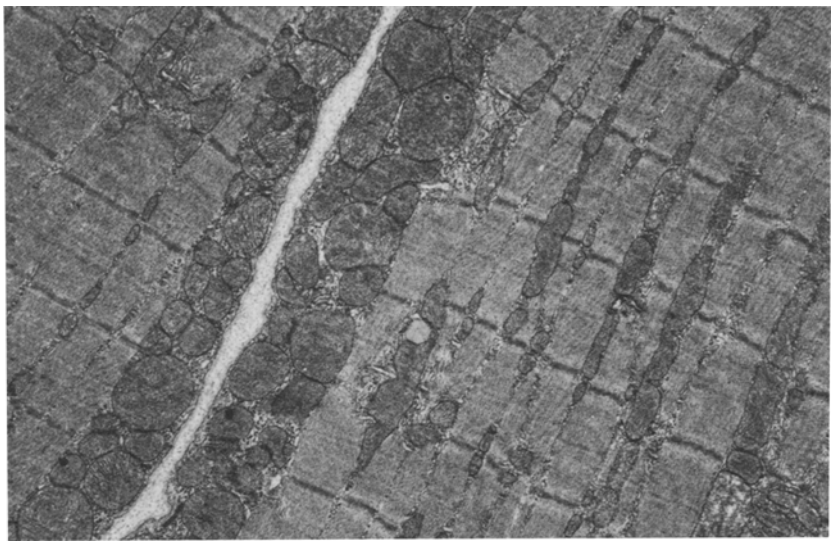


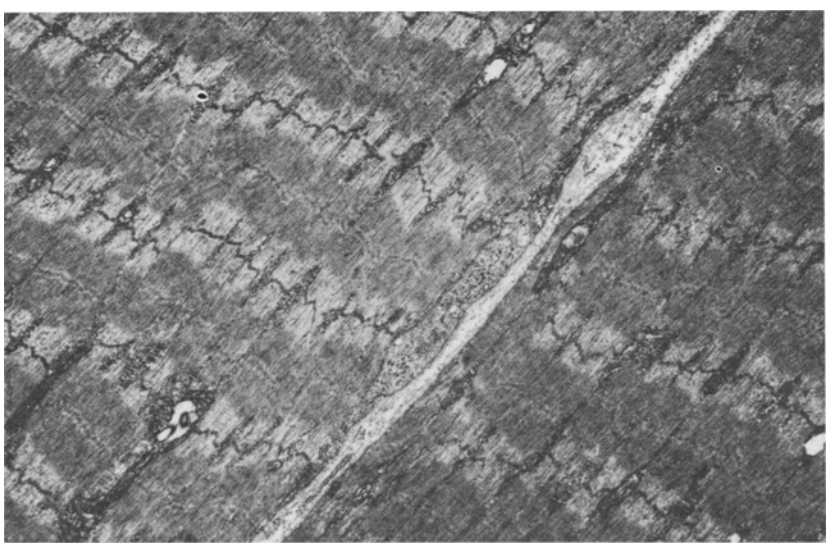
Figure 1b. Cross-section of the semitendinosus muscle of a cold-acclimated rat. Stained for succinic dehydrogenase × 15.

The ultrastructure of the red and white fibers of the muscle of the control rats corresponded to the description of Gauthier [18, 19]. In the red fiber, there are subsarcolemmal aggregates of large, fat mitochondria with a moderate number of cristae and an expanded matrix (Fig. 2A). Similar large mitochondria occur between the myofibrils, often in pairs at the I bands but also frequently extending one sarcomere in length. In the white fiber of the control rats (Fig. 2B) the mitochondria are generally fewer in number and there are few subsarcolemmal aggregations. Mitochondria occur relatively infrequently in pairs between the myofibrils at the I bands and occasionally a long mitochondrion is seen (Fig. 2B).

The ultrastructure of the fibers in the red and white portions of the semitendinosus muscle of the cold-acclimated rats differs in certain respects from that of the control rats. In the red fibers the mitochondria are small in diameter, although they may be very long and thin (Fig. 3A). The aggregations in the subsarcolemmal region are less conspicuous but there are numerous small mitochondria between the myofibrils, usually in pairs at the I bands. The fibers in the white portion of the semitendinosus

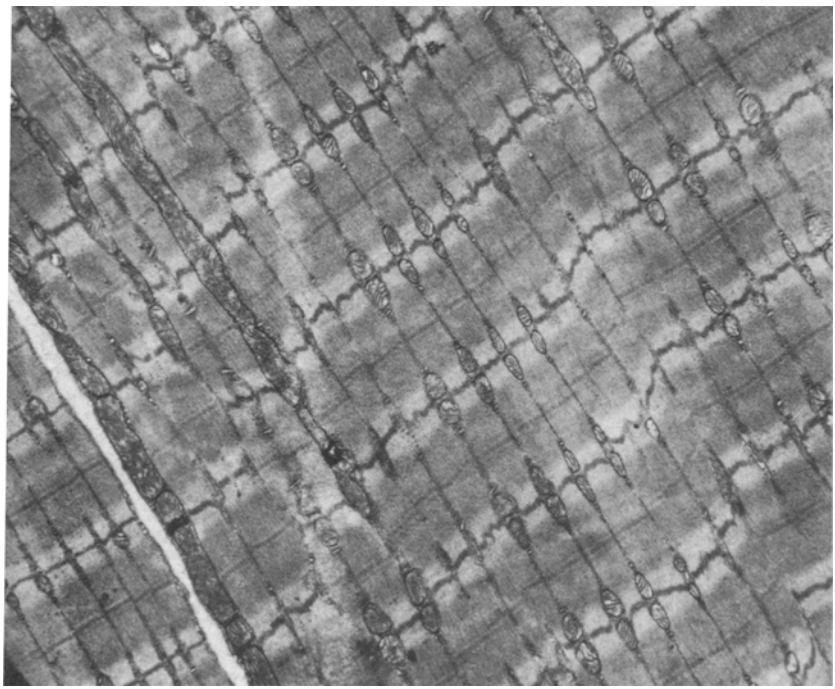


2a



2b

Figure 2. Electron micrograph of fibers in (A) the red portion and (B) the white portion of the semitendinosus muscle of a warm-acclimated rat ×14,000. The red fiber (A) shows the characteristic subsarcolemmal aggregation of large mitochondria and the large interfibrillar mitochondria. The white fiber (B) shows that characteristic absence of subsarcolemmal aggregates of mitochondria and paucity of mitochondria in the interior of the fibers.

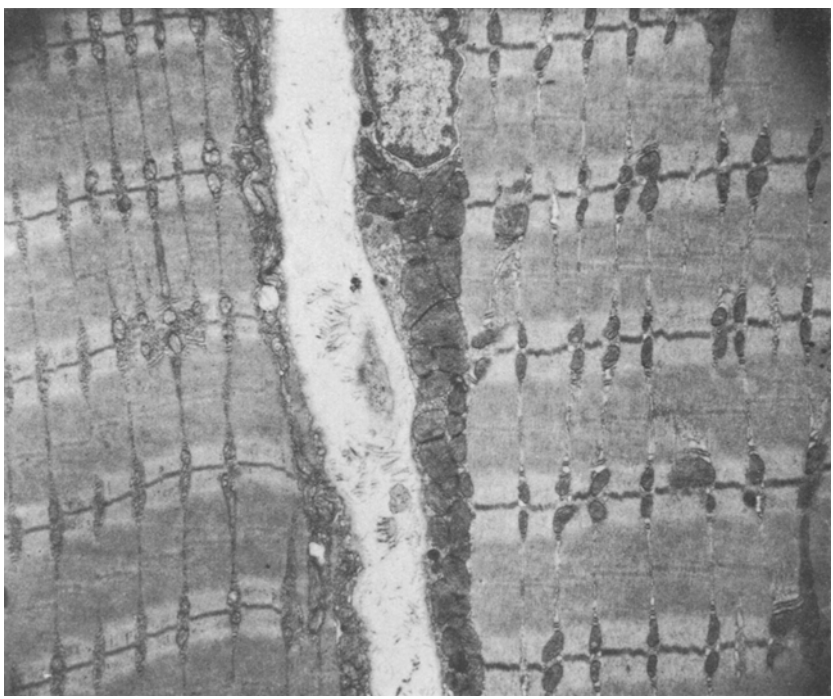


3a

Figure 3. Electron micrographs of fibers in (A) the red portion and (B) the white portion of the semitendinosus muscle of a cold-acclimated rat. The rat had been acclimated to cold for four weeks (× 14,000). The subsarcolemmal aggregates of mitochondria are smaller than in the red fiber of the warm-acclimated rat (A) (see Fig. 2A) and are usually composed of a single row of long thin mitochondria. The interior of the fiber contains numerous small paired mitochondria situated at the I bands. Some very long mitochondria are occasionally seen, as the one in this photograph which appears to be more than 3 sarcomeres in length. In the

of the cold-acclimated rats no longer have the appearance characteristic of white fibers (Fig. 3B). They are rich in small mitochondria, particularly in the interfibrillar region; these mitochondria are occasionally one sarcomere in length.

The pictures shown are representative of more than 100 sections from different blocks of tissues from 12 different animals. Nevertheless, the impressions described above are subjective impressions. In a tissue with a highly organized structure like muscle it is difficult to show single, or indeed several, sections that would be truly representative of the complete structure of the muscle. Although methods are available for quantitative determination from electron micrographs of size, volume and number of mitochondria in tissues with a random structure such as liver [31] these



3b

white fiber of the cold-acclimated rat (B) there are usually few subsarcolemmal aggregates of mitochondria but the interior of the fiber contains a large number of small mitochondria. These mitochondria occasionally appear to be 1 sarcomere in length. The fiber in this photograph appears to resemble an intermediate fiber both in the distribution of mitochondria and in the width of the Z bands. However, this is the predominant fiber in the white portion of the semitendinosus muscle of the cold-acclimated rat.

methods are not readily applicable to tissues with a nonrandom structure such as skeletal muscle. We therefore sought another method which would permit a quantitative estimation of the number and size of mitochondria in skeletal muscle and would allow us to assess the correctness of the impressions gained from the electron micrographs. The method chosen was that of Baranowicz [26] which, by assessing the ratio of red cells and mitochondria in smears obtained from suspensions containing a known number of red cells and an unknown number of mitochondria, permits the calculation of the number of mitochondria. Protein and enzyme determinations then permit the calculation of the protein and enzyme content of one mitochondrion and thus a measure of its size.

The most notable result obtained in this experiment is the finding of an

TABLE I. Counting of mitochondria isolated from red and white portions of the semitendinosus muscle of cold-acclimated and control rats

		Control	Cold-acclimated	P^{*a}
Number of mitochondria $\times 10^{-9}$/g muscle	Red	65.3 ± 4.91	108.8 ± 8.54	<0.005
	White	38.9 ± 4.31	59.9 ± 8.29	<0.10
	P^{**b}	<0.01	<0.01	
Protein content (pg/mitochondrion)	Red	0.111 ± 0.005	0.062 ± 0.003	<0.001
	White	0.114 ± 0.006	0.073 ± 0.007	<0.005
	P^{**}	N.S.	N.S.	
Mitochondrial number/mg protein $\times 10^{-9}$	Red	9.09 ± 0.41	16.41 ± 0.89	<0.001
	White	8.81 ± 0.42	14.05 ± 1.30	<0.001
	P^{**}	N.S.	N.S.	
Cytochrome oxidase μatoms O_2/ mitochondrion $\times 10^9$	Red	1.98 ± 0.11	1.17 ± 0.04	<0.001
	White	1.63 ± 0.08	1.08 ± 0.12	<0.01
	P^{**}	<0.05	N.S.	
Specific activity of cytochrome oxidase (μatoms O_2/mg protein)	Red	18.12 ± 1.67	19.14 ± 1.03	N.S.
	White	14.31 ± 0.63	14.87 ± 1.31	N.S.
	P^{**}	N.S.	<0.05	

Total mitochondrial protein (mg/g muscle)	Red	7.26 ± 0.75	6.66 ± 0.51	N.S.
	White	4.38 ± 0.29	4.35 ± 0.62	N.S.
	P^{**}	<0.02	<0.05	
Cytochrome oxidase (μatoms O_2/g muscle)	Red	128.2 ± 5.07	126.4 ± 7.59	N.S.
	White	63.0 ± 6.75	62.2 ± 4.55	N.S.
	P^{**}	<0.001	<0.001	
Recovery of mitochondrial protein (mg/g muscle)	Red	2.97 ± 0.40	2.18 ± 0.23	N.S.
	White	1.17 ± 0.06	1.22 ± 0.15	N.S.
	P^{**}	<0.005	<0.02	
Recovery of cytochrome oxidase (%)	Red	40.92 ± 2.68	32.86 ± 3.04	N.S.
	White	26.95 ± 2.15	28.74 ± 1.85	N.S.
	P^{**}	<0.01	N.S.	

[a] P^{*} denotes significance of the difference between control and cold-acclimated muscle.
[b] P^{**} denotes significance of the difference between red and white muscle from the same group of rats ($n=4$).

increase in the number of mitochondria per gram of muscle in the cold-acclimated rat (Table I). The increase occurs in both red and white muscle, being larger in the red muscle and barely significant in the white muscle. The total mitochondrial mass per gram of muscle is, however, unchanged, as shown by the unchanged total cytochrome oxidase activity and amount of mitochondrial protein per gram of muscle. The total unchanged mitochondrial mass is, therefore, divided up into smaller packages, as shown by the reduced protein content and reduced cytochrome oxidase content of each mitochondrion. It is concluded that the mitochondria of the red and white muscle of the cold-acclimated rat are smaller and more numerous. This is in agreement with the impression gained from the morphological studies.

In order to determine whether the increase in number and decrease in size of the muscle mitochondria was associated with the existence of the adaptation for nonshivering thermogenesis and of the enhanced metabolic response to noradrenaline two experimental approaches were used. First, cold-acclimated rats were returned to room temperature. As expected the enhanced metabolic response to noradrenaline decreased during the subsequent two weeks (Table II). The increased number of mitochondria also decreased and was not significantly greater than the number in the muscle of the control rats (Table II). The ultrastructural appearance of the red and white fibers of the semitendinosus of the cold-acclimated rats that had undergone two weeks of deacclimation was indistinguishable from that of the control rats.

Thus, the altered mitochondria are associated with the existence of the enhanced metabolic response to noradrenaline. However, it remains possible that the alteration in the mitochondria is secondary to the continuously high metabolic rate, which subsides when the cold-acclimated rate are returned to room temperature. The second experimental approach, namely, treatment of the rats with oxytetracycline during acclimation to cold, was designed to prevent the development of the enhanced metabolic response to noradrenaline while permitting a continuously high metabolic rate in the cold. That the development of the enhanced metabolic response to noradrenaline was prevented by treating the rats with oxytetracycline is shown in Table III. The treatment almost entirely prevented the increase in number and decrease in size of the muscle mitochondria of the cold-acclimated rats (Table IV). The oxytetracycline-treated rats had as high a metabolic rate in the cold (4.26 ± 0.15 ml oxygen/100 cm^2 min, $n=5$) as did the saline-treated cold-acclimated rats 4.46 ± 0.16 ml oxygen/100 cm^2 min, $n=5$). Thus the increase in number and decrease in size of the mitochondria is not secondary to a high metabolic rate but is associated with the enhanced metabolic response to noradrenaline.

TABLE II. Counting of mitochondria in mixed leg and back muscle of cold-acclimated rats during deacclimation

	Week[a]	Control	Cold→Warm	P[b]
Number of mitochondria	0	80.6 ± 2.13	155.9 ± 14.24	<0.01
× 10^{-9}/g muscle	1	103.7 ± 7.50	165.8 ± 6.65	<0.005
	2	95.4 ± 9.21	115.4 ± 7.01	N.S.
Protein content (pg/	0	0.125 ± 0.004	0.078 ± 0.004	<0.005
mitochondrion)	1	0.119 ± 0.004	0.098 ± 0.004	<0.02
	2	0.137 ± 0.018	0.125 ± 0.014	N.S.
Mitochondrial number/mg	0	8.04 ± 0.27	12.93 ± 0.65	<0.02
protein × 10^{-9}	1	8.41 ± 0.27	10.28 ± 0.40	<0.02
	2	7.48 ± 0.86	8.20 ± 0.84	N.S.
Cytochrome oxidase (μatoms	0	2.28 ± 0.22	1.26 ± 0.09	<0.02
O_2/mitochondrion × 10^9)	1	1.74 ± 0.10	1.12 ± 0.09	<0.01
	2	1.89 ± 0.08	1.51 ± 0.07	<0.025
Specific activity of cytochrome	0	18.37 ± 2.19	16.28 ± 1.70	N.S.
oxidase (μatoms O_2/mg	1	14.54 ± 0.44	11.44 ± 0.76	<0.025
protein)	2	14.02 ± 1.33	12.42 ± 1.53	N.S.
Total mitochondrial protein	0	10.06 ± 0.57	12.19 ± 1.59	N.S.
(mg/g muscle)	1	12.33 ± 0.61	16.23 ± 1.24	<0.05
	2	12.83 ± 0.48	14.57 ± 2.16	N.S.
Cytochrome oxidase (μatoms	0	177.6 ± 11.8	193.0 ± 3.6	N.S.
O_2/g muscle)	1	179.8 ± 11.9	184.6 ± 19.2	N.S.
	2	179.8 ± 17.0	173.7 ± 5.1	N.S.
Recovery of mitochondrial	0	3.44 ± 0.49	2.70 ± 0.32	N.S.
protein (mg/g muscle)	1	4.89 ± 0.46	3.48 ± 0.49	N.S.
	2	3.63 ± 0.21	3.68 ± 1.09	N.S.
Recovery of cytochrome	0	34.58 ± 2.36	22.64 ± 3.18	<0.05
oxidase (%)	1	40.05 ± 5.04	21.36 ± 1.58	<0.025
	2	28.28 ± 1.12	24.61 ± 3.93	N.S.
Metabolic response to	0	53.3 ± 3.51	93.9 ± 3.56	<0.001
noradrenaline (ml O_2/100 cm^2	1	43.2 ± 6.16	67.4 ± 3.93	<0.02
× 30 min)	2	46.1 ± 3.68	51.0 ± 4.15	N.S.

[a] Week of deacclimation.

[b] *P* denotes the significance of the difference between the rats undergoing deacclimation (Cold→Warm) and the control rats ($n = 3$).

Treatment with oxytetracycline did not have any major effect on the ultrastructure of the red and white fibers of the semitendinosus of the warm-acclimated rats, although a few mitochondria appeared to have lost some of their internal organization, particularly in the red fibers. The ultra-structure of red and white fibers of the semitendinosus of the saline-treated cold-acclimated rats showed the expected increase in number of

TABLE III. Effect of treatment with oxytetracycline on the metabolic response to noradrenaline of rats during and after acclimation to cold (metabolic response to noradrenaline: ml O_2/100 cm^2 x 30 min)

Week	S[a]	S-T	T	T-S
0	15.1 ± 1.80 (5)		18.4 ± 3.70 (5) N.S.[b]	
2	55.0 ± 5.70 (4)		28.7 ± 3.10 (3) <0.02	
3	50.7 ± 1.24 (5)	41.2 ± 2.46 (5) <0.01	30.3 ± 5.20 (4) <0.005	58.0 ± 3.7 (4) N.S.
4	55.2 ± 2.60 (5)	39.2 ± 1.37 (4) <0.005	28.7 ± 2.23 (5) <0.001	65.9 ± 0.6 (4) <0.01

[a] Abbreviations are: S, saline-treated rats; T, oxytetracycline-treated rats; these two groups of rats were treated during the first four weeks of acclimation to cold; S-T, rats treated with oxytetracycline during their third and fourth weeks in the cold only; T-S, rats treated with oxytetracycline during the first two weeks in the cold and with saline during the third and fourth weeks in the cold. Values in parentheses show number of animals in each group.
[b] Probability of a significant difference between the designated group and group S.

mitochondria. The reduction in mitochondrial size was not as marked as previously observed, possibly because these rats had been in the cold for only two weeks and were, therefore, only partially cold-acclimated; some large mitochondria were still seen in the red fibers. Treatment of the rats with oxytetracycline during the two weeks of acclimation to cold prevented the increase in number of mitochondria. In these rats many mitochondria appeared swollen and mitochondria with cristae arranged in concentric rings were commonly observed.

Not only can the development of the alteration in the muscle mitochondria and the development of the enhanced metabolic response to noradrenaline be prevented by treatment of the rats with oxytetracycline during acclimation to cold but both can be reversed if the treatment with oxytetracycline is started only after acclimation to cold has occurred. The reversal of the enhanced metabolic response to noradrenaline is illustrated in Table III and the reversal of the increased number of mitochondria in muscle is illustrated in Table V. It should be noted that although the size of the mitochondria increases in the oxytetracycline-treated cold-acclimated rats, as indicated by an increased protein content per mitochondrion, the cytochrome oxidase content per mitochondrion does not increase significantly, probably because mitochondrial protein synthesis is required for its formation and is inhibited in these animals.

TABLE IV. Counting of mitochondria isolated from mixed leg and back muscle of cold-acclimated and control rats: Effect of treatment with oxytetracycline during acclimation to cold[c]

	Control Rats			Cold-Acclimated Rats		
	Saline	Oxytetracycline	P^a	Saline	Oxytetracycline	P^a
Number of mitochondria $\times 10^{-9}$/g muscle	65.6 ± 3.57	60.7 ± 5.27	N.S.	110.1 ± 6.86 <0.001[b]	64.9 ± 4.22 N.S.[b]	<0.001
Protein content(pg/mitochondrion)	0.139 ± 0.01	0.124 ± 0.01	N.S.	0.088 ± 0.008 <0.001	0.144 ± 0.005 N.S.	<0.05
Mitochondrial number/mg protein $\times 10^{-9}$	7.36 ± 0.53	8.13 ± 0.58	N.S.	11.8 ± 1.34 <0.02	8.89 ± 0.43 N.S.	N.S.
Cytochrome oxidase (μatoms O_2/mitochondrion $\times 10^9$)	2.42 ± 0.24	2.05 ± 0.08	N.S.	1.55 ± 0.11 <0.02	1.92 ± 0.13 N.S.	N.S.
Specific activity of cytochrome oxidase (μatoms O_2/mg protein)	17.79 ± 2.38	16.82 ± 1.70	N.S.	18.61 ± 2.99 N.S.	17.24 ± 2.06 N.S.	N.S.
Total mitochondrial protein (mg/g muscle)	9.14 ± 0.97	7.67 ± 0.95	N.S.	9.65 ± 1.06 N.S.	7.41 ± 0.68 N.S.	N.S.
Cytochrome oxidase (μatoms O_2/g muscle)	157.1 ± 13.5	123.9 ± 9.9	N.S.	172.7 ± 12.1 N.S.	123.2 ± 9.0 N.S.	<0.02
Recovery of mitochondrial protein (mg/g muscle)	3.10 ± 0.22	2.22 ± 0.41	N.S.	2.91 ± 0.22 N.S.	2.03 ± 0.24 N.S.	<0.05
Recovery of cytochrome oxidase (%)	34.8 ± 3.01	29.2 ± 3.70	N.S.	30.3 ± 2.51 N.S.	27.2 ± 1.20 N.S.	N.S.

[a] Probability of a significant difference between saline-treated and oxytetracycline-treated rats in the designated group ($n = 5$).
[b] Probability of a significant difference between the cold-acclimated rats and the corresponding control rats ($n = 5$).
[c] The cold-acclimated rats correspond to those designated group S (2 weeks) and group T (2 weeks) in Table III.

TABLE V. Counting of mitochondria isolated from mixed leg and back muscle of cold-acclimated rats: Effect of treatment with oxytetracycline after acclimation to cold [a]

	Saline	Oxytetracycline	P [b]
Number of mitochondria $\times 10^{-9}$/g muscle	95.0 ± 7.18	64.4 ± 7.43	<0.05
Protein content (pg/mitochondrion)	0.094 ± 0.002	0.141 ± 0.007	<0.005
Mitochondrial number/mg protein $\times 10^{-9}$	10.42 ± 0.30	7.15 ± 0.39	>0.005
Cytochrome oxidase (μatoms O_2/mitochondrion $\times 10^9$)	1.57 ± 0.01	1.89 ± 0.13	N.S.
Specific activity of cytochrome oxidase (μatoms O_2/mg protein)	16.68 ± 0.064	13.48 ± 0.76	<0.05
Total mitochondrial protein (mg/g muscle)	8.82 ± 0.44	9.27 ± 0.87	N.S.
Cytochrome oxidase (μatoms O_2/g muscle)	147.6 ± 1.9	124.2 ± 6.6	<0.05
Recovery of mitochondrial protein (mg/g muscle)	3.69 ± 0.19	3.34 ± 0.52	N.S.
Recovery of cytochrome oxidase (%)	41.74 ± 2.38	36.23 ± 5.40	N.S.

[a] These rats correspond to those designated group S (week 4) and group S-T (week 4) in Table III.

[b] Probability of a significant difference between oxytetracycline-treated rats and saline-treated rats ($n = 3$).

Discussion

The conclusion drawn from the structural studies and from the counting of mitochondria isolated from skeletal muscle is that both red and white muscle fibers of the cold-acclimated rat contain a greater number of mitochondria. Although there are more mitochondria, the size of the mitochondria is reduced to such an extent that the total mitochondrial mass in the muscle is unchanged. Thus the alteration is a repackaging of mitochondrial material into smaller units rather than a change in the quantity of mitochondrial material.

Previous studies have also reported increases in coenzyme Q content [32], cytochrome c content [33], succinoxidase activity and activities of succinic dehydrogenase and malate dehydrogenase [34, 35] in muscle of cold-acclimated rats. Some authors have, however, found no change in

succinic dehydrogenase activity [36] and have reported that while an increase in cytochrome c content occurs in some muscles, e.g. abdominal muscles, it does not occur in all and the total cytochrome c content of the cold-acclimated rat is not altered [37]. We find that the cytochrome oxidase concentration in red and white portions of the semitendinosus and in mixed leg muscle is unaltered in the cold-acclimated rat.

In the intact cold-acclimated rat the change in the mode of heat production which occurs in muscle is a switch from shivering thermogenesis to nonshivering thermogenesis. It is not the capacity of the rat to consume oxygen which is altered but the way in which that capacity is evoked. In the shivering rat it is evoked by the stimulation of the motor nerves to the muscle which results in shivering. In the rat which is not shivering but raising its metabolic rate by nonshivering thermogenesis it is evoked by the action of noradrenaline on the skeletal muscle which results, via an unknown mechanism, in an increase in the metabolic rate of the muscle. Thus, the unaltered mitochondrial mass in the skeletal muscle of the cold-acclimated rat would be consistent with the unaltered capacity of the rat to consume oxygen. The altered packaging of the mitochondrial material of the skeletal muscle of the cold-acclimated rat into smaller units is presumably a reflection of an altered regulatory mechanism in these mitochondria responsible for the appearance of the capacity for nonshivering thermogenesis.

The changes we see in the muscle of the cold-acclimated rat are seen in an animal which has undergone intense shivering for an extended period before full acclimation to cold is achieved. It might be argued that these changes are secondary to the shivering rather than being associated with the development of the capacity for nonshivering thermogenesis. However, the changes are different from those which result from prolonged muscle activity. Exercise-training in rats results in an increased proportion of red fibers in the muscle [38, 39], an increase in the number and size of mitochondria [40] and an increase in the content of cytochrome oxidase. Moreover, trained rats do not have an enhanced metabolic response to noradrenaline [41]; they are slightly better at increasing their oxygen uptake during cold-stress, possibly because of a greater capacity for shivering [41]. Whereas training causes the appearance of more and larger mitochondria with an increased mitochondrial mass per unit weight of muscle, acclimation to cold is associated with the appearance of more but smaller mitochondria with an unchanged mitochondrial mass per unit weight of muscle. A report of increased mitochondrial enzyme activities (cytochrome oxidase and succinic dehydrogenase) in muscles of cold-acclimated rats, at variance with our findings, is probably due to the method used to acclimate the rats which were immersed daily in cold water

(15°C) and continued to shiver violently each day during the treatment throughout the period of acclimation [42, 43].

We have, nevertheless, tried to distinguish between changes associated with cold-acclimation and changes secondary to shivering by studying these changes in a number of different circumstances in which the adaptive state of the animal has been altered by preventing the development of the adaptation for nonshivering thermogenesis or by reversing the adaptation after it has already occurred. The alteration in the muscle mitochondria does not occur when the development of the enhanced response is inhibited with oxytetracycline, disappears when the enhanced response once developed in the cold-acclimated rat is reversed with oxytetracycline, and disappears when the rat undergoes deacclimation after return to room temperature. That the alteration is not secondary to shivering or to a high metabolic rate can be concluded from its failure to occur in the oxytetracycline-treated rats which have a high metabolic rate in the cold and have been subjected to shivering.

The inhibition of the appearance of the altered mitochondria in the rats treated with oxytetracycline during the first two weeks of acclimation to cold would suggest that mitochondrial protein synthesis is necessary for the alteration in muscle mitochondria to occur. The reversal of the alteration which occurs in cold-acclimated rats by treatment with oxytetracycline while they continue to live in the cold would suggest that mitochondrial protein synthesis is also necessary for the maintenance of the alteration. Moreover, the half-lives of some groups of mitochondrial proteins are decreased in skeletal muscle of cold-acclimated rats [15]. We assume, therefore, that the repackaging of mitochondrial material of skeletal muscle into smaller units which occurs during cold-acclimation involves an alteration in mitochondrial protein synthesis.

Hyperthyroidism is another condition in which skeletal muscle mitochondria are known to be altered. There appear to be more and larger mitochondria in the muscle [44], an increase in the content of mitochondrial enzymes in the muscle [45], and an increased respiratory capacity of the isolated mitochondria in the presence of a variety of substrates [45]. The mitochondria are not uncoupled, having a normal P/O ratio and respiratory control index [45]. However, the structural change in the mitochondria does not disappear when the hyperthyroid state reverts to the euthyroid state and the increased respiratory capacity of the isolated mitochondria decreases to the normal level [44]. Thus, the functional significance of the structural alteration is not clear.

A human metabolic disorder characterized by a high metabolic rate not due to hyperthyroidism is also associated with altered muscle mitochondria [46]. The mitochondria of the muscle are more numerous

and larger and present many bizarre forms. They have an increased specific activity of cytochrome oxidase [46] and are loosely coupled [47]. A second case reported recently [48] was treated with chloramphenicol which reduced both the elevated metabolic rate and the increased number of mitochondria and increased mitochondrial enzyme activity. The effect of chloramphenicol, an inhibitor of mitochondrial protein synthesis, indicates that mitochondrial protein synthesis is necessary for the maintenance of the abnormal state of the mitochondria and is presumably altered in the disorder.

These three other conditions in which muscle mitochondria have reported to be altered differ from the cold-acclimated state, in which muscle mitochondria are also altered. The functional change with which the alteration is associated is different in each case. In hyperthyroidism and the Luft hypermetabolic state the basal metabolic rate is increased. In the exercise-trained rats the basal metabolic rate is normal but the capacity to raise metabolic rate during exercise is greater. In the cold-acclimated rats the basal metabolic rate is unaltered or only slightly increased, the capacity to raise metabolic rate during exercise is normal [49], but the capacity to raise metabolic rate in response to stimulation by noradrenaline is greatly elevated. It is obvious that the alteration in the function of muscle mitochondria in these four conditions, if it is based upon an altered mitochondrial structure and composition, should be different in the four. The only one in which the altered function can logically be attributed to the alteration in the muscle mitochondria is exercise-training. For the others, the relation of the altered function to the altered form of the mitochondria will require a more detailed molecular dissection of the mitochondria than has so far been done. In the cold-acclimated rat, a subtle change in the regulatory mechanisms which govern mitochondrial function, particularly the coupled state of the mitochondria, would be a likely basis for the functional change which is responsible for the greatly increased capacity to respond to noradrenaline by an increase in metabolic rate and hence for the adaptation for nonshivering thermogenesis.

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