ADAPTATION OF MUSCLE MITOCHONDRIA IN COLD-ACCLIMATION.

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A thesis submitted to the

School of Graduate Studies

of the

University of Ottawa.

In partial fulfilment of the requirements

for the degree of Doctor of Philosophy.

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University of Ottawa.

August 1973.

To RAQUEL , GLORIA AND VANESSA.

ACKNOWLEDGEMENTS.

I wish to express my gratitude to Dr.Jean Himms-Hagen. I deeply appreciate the manner in which Dr.Himms-Hagen has intellectually and materially supported this investigation.

I would also like to thank Dr.J.Metuzals for the use of the electron microscope facilities and to Mr.W.Redmond and Mr.Chow-Chong for their technical assistance in the different aspects of electron microscopy. I am grateful to Dr.L.F.Belanger of the Department of Histology for the use of the histochemical facilities and to Dr.R.Narbaitz for his advice in the histochemical procedures.

Lastly,and in a very special manner,I thank to Mrs. A.Inglis for her skilful technical assistance.

ABBREVIATIONS.

ADP	-	adenosine-5'-diphosphate.
ATP	-	adenosine-5'-triphosphate.
cyclic AMP	-	adenosine-3',5'-monophosphate.
EDTA	-	ethylene diamine tetraacetic acid.
IBAT	-	interscapular brown adipose tissue.
NST	-	nonshivering thermogenesis.
SNS	-	sympathetic nervous system.
TRIS	-	tris-hydroxymethyl-aminomethane.

SUMMARY.

When homeothermic animals are placed in a cold environment, they increase their heat production by shivering in order to maintain their body temperature. After having been in the cold about 4 weeks they undergo a change that enables them to maintain their body temperature without shivering. Such rats are said to be cold-acclimated and the term nonshivering thermogenesis is used to describe the processes whereby they increase their heat production.

The metabolic processes involved in the increase in heat production due to shivering are the same as those involved in the increase in heat production during exercise. In contrast, the biochemical nature and mechanism of nonshivering thermogenesis are not understood. It is known that nonshivering thermogenesis is an enhanced calorigenic response to the noradrenaline secreted by the animal and that the major part of nonshivering thermogenesis occurs in skeletal muscle. No biochemical differences between muscles of cold-acclimated rats and muscles of warm-acclimated control rats have hitherto been found which might explain the nature of the adaptation which leads to the increased capacity for nonshivering thermogenesis.

Two recent findings in this laboratory have suggested a special role for mitochondrial protein synthesis in adaptation to cold. Since muscle mitochondrial protein synthesis appears to be involved in the adaptation to cold it seems likely that this would be reflected in a change in the morphology and / or properties of the mitochondria of the skeletal muscle. The adaptation for nonshivering thermogenesis may well be associated with structural changes in the muscle mitochondria.

The principal purpose of the research described in this thesis was to find either biochemical or morphological changes, or both, in skeletal muscle mitochondria of cold-acclimated rats and to correlate such changes with the occurence of nonshivering thermogenesis.

A comparison of both red and white muscles of cold-acclimated rats with both red and white muscles of warm-acclimated control rats has been made in the following ways:

a) Electron microscopy of red and white muscles.

b) Fiber characteristics and distribution in red and white muscles.

c) Measurement of number, size and various enzyme activities of mitochondria isolated from muscle.

These procedures were carried out on rats that were undergoing acclimation to cold, or undergoing deacclimation to cold. In addition, conditions were studied in which the normal acclimation process was modified: by prevention of the acclimation to cold with oxytetracycline, by reversal of the acclimation to cold with oxytetracycline, by acceleration of the deacclimation to cold by removal of the interscapular brown adipose tissue.

In these experiments, a marked difference between mitochondria of muscles of cold-acclimated rats and mitochondria of muscles of warm-acclimated rats has been seen. The principal differences seen are:

1. An increase in the number and a reduction in the size of the mitochondria in both red and white muscle.

2. An increase in the proportion of red fibers and an apparent transformation of white fibers into fibers which appear more like intermediate fibers.

All the changes described were prevented or reversed when the adaptation to cold was prevented or reversed by the means indicated suggesting that the adaptive changes in the muscle mitochondria of cold-acclimated rats are associated with the development and maintenance of nonshivering thermogenesis.

This is the first biochemical difference between muscles of cold-acclimated rats and muscles of warm-acclimated rats to be described. The way in which the alteration in number and size of muscle mitochondria is related to the altered function of these mitochondria in nonshivering thermogenesis is not yet understood. However, these findings indicate that further study of mitochondria of skeletal muscle of cold-acclimated rats is likely to shed some light on the mechanism of nonshivering thermogenesis.

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CHAPTER I: PURPOSE OF THE RESEARCH

The principal purpose of the research described in this thesis is to find either biochemical or morphological changes, or both, in skeletal muscle mitochondria of cold-acclimated rats and to correlate such changes with the occurrence of nonshivering thermogenesis in these animals.

Nonshivering thermogenesis is an increase in metabolic rate which occurs in response to exposure to cold and in the absence of shivering. Nonshivering thermogenesis is known to result from an enhanced calorigenic response to the noradrenaline and adrenaline secreted by the animal as a consequence of the cold stress. The importance of skeletal muscle in the process of nonshivering thermogenesis has been well documented. It is known that skeletal muscle is the major site of heat production during the development and maintenance of the cold-acclimated state.

Despite extensive investigation the biochemical nature of nonshivering thermogenesis is unknown: no changes in muscle structure or function have been found, except the change from shivering thermogenesis to nonshivering thermogenesis. However, it can be assumed that there must be a change in skeletal muscle responsible for the development of the capacity for nonshivering thermogenesis. The change is not detectable by measuring known

biochemical reactions. Recent findings in this laboratory have suggested an altered mitochondrial protein synthesis in acclimation to cold. It seems likely that altered mitochondrial protein synthesis would be reflected in a change in the appearance of the muscle mitochondria. It is obvious that nonshivering thermogenesis occurs in the mitochondria and it might well be based on a change in the structure and function of these organelles.

> A subsidiary aim of this research is to provide further evidence for an endocrine function of brown adipose tissue in the development and maintenance of the cold-acclimated state by studying the role of this tissue in the maintenance of the change in skeletal muscle mitochondria in the cold-acclimated animal.

Brown adipose tissue is known to have not only a heat-producing function in the cold-acclimated animal but also a function in maintaining the adaptive state responsible for the capacity of the animal to use nonshivering thermogenesis. It has been postulated that brown adipose tissue secretes a factor that influences the capacity of other tissues, particularly skeletal muscle, to perform nonshivering thermogenesis, i.e., to respond to noradrenaline with a large increase in metabolic rate.

The review of the literature presented in Chapter II provides a background for the several aspects of this research.

Sections A - D describe the nature of cold-acclimation and present the evidence for muscle as a major site of nonshivering thermogenesis. Section E considers the special roles of brown adipose tissue in cold-acclimation and Section F discusses current concepts of the nature of nonshivering thermogenesis. As a background to the morphological studies a brief review of the ultrastructure of skeletal muscle is presented in Section G. Other studies in which attempts have been made to correlate changes in muscle mitochondria with changes in muscle function are reviewed in Section H.

CHAPTER II: INTRODUCTION

Mammals are homeotherms. This term implies that the body temperature is kept within certain limits. Therefore, when a homeothermic animal is placed in a cold environment it is faced with the problem of a rapid loss of heat and a consequent lowering of body temperature. Homeothermic animals have developed several different mechanisms to increase their heat production or to decrease their heat loss.

The regulatory mechanisms by which the animal reduces heat loss includes piloerection and peripheral vasoconstriction, responses which are both mediated by the activation of the sympathetic nervous system (SNS) produced by the exposure to cold. These mechanisms will be not reviewed here.

The regulatory mechanisms by which the animal increases heat production include the increase in metabolic rate produced by shivering thermogenesis and the increase in metabolic rate produced by means other than shivering or other muscle movements, usually referred to as nonshivering thermogenesis. These mechanisms will be reviewed here in some detail, with special reference to nonshivering thermogenesis which is of greatest importance during long periods of exposure to cold when the animal can be considered to be cold-acclimated.

Definitions

It is necessary at this point to define some of the terms used in this thesis:

- "Cold-exposure" means the relatively brief (hours or up to 2 days) exposure of an animal to a temperature lower than that to which it has been accustomed.
- "Cold-acclimated" are those animals which have been kept at low temperature (4°C is commonly used) for long periods of time, several weeks or months. These animals adapt to living in the cold and maintaining a high metabolic rate.
- "Cold-acclimation" is the adaptation undergone by the cold-acclimated animal. Various adaptive adjustments occur during the acclimation period in which shivering thermogenesis is replaced by nonshivering thermogenesis.
- "Deacclimation" is the slow reversal of the cold-acclimated state, usually achieved by putting back to room temperature the cold-acclimated animal.
- "Warm-acclimated" are the control animals which have been kept at usual laboratory temperature (25-28°C).

Among the several species used in studies of cold-acclimation the rat adapts most readily to the cold; also the rat has been the species most extensively studied. For these reasons this thesis will consider only the rat unless another species is specifically indicated.

Section A: COLD EXPOSURE AND SHIVERING THERMOGENESIS

Hemingway (1963,1968) defined shivering as a rhythmic involuntary contraction of voluntary muscle which is activated as a response to cold and is under the control of the central nervous system.

Shivering then can be classified as an emergency mechanism for homeotherms; its role is the production of additional metabolic heat. When the animal is transferred from a warm confortable environment to a cold environment its oxygen consumption rate is increased. As shown by different authors, this increase can be up to a maximum value of 5 times the basal metabolic rate, depending on the species and the experimental conditions (Hemingway, 1963).

Since shivering is a rhythmic muscle contraction the metabolic effects of shivering resemble those of light exercise. Voluntary activity can increase the oxygen consumption rate in the same manner as shivering and careful attention must be paid to experimental details if separation of shivering from voluntary motor activity is to be made.

Héroux, Hart and Depocas (1956) studied the metabolism and muscle activity of anesthetized warm- and cold-acclimated rats on exposure to cold. The same group (Hart, Héroux and Depocas, 1956) also conducted similar experiments in unanesthetized rats. In both cases, with slight differences, it was found that the warm-acclimated rat exposed to 6°C shows a 30-50% increase in oxygen consumption; the electromyograms from leg and back muscles showed a marked and continuous rise in electrical output (Fig. 1). They also showed that the electrical activity of the muscle increased in the warm-acclimated rats as the environmental temperature was lowered (Fig. 2). However it was not directly proportional to the increase in metabolic rate (Depocas, 1956).

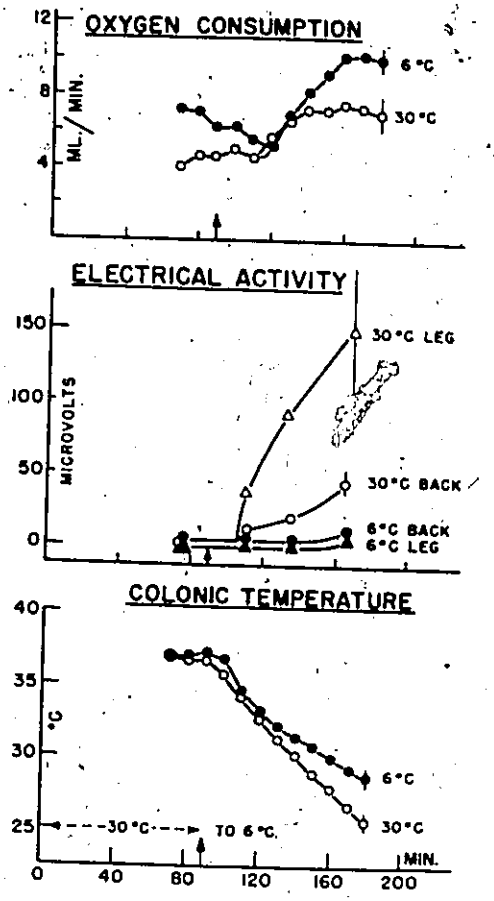

Figure 1. Mean oxygen consumption, muscle electrical activity and colonic temperature of anaesthetized warm-acclimated and cold-acclimated rats, exposed to 6°C. Standard errors of means (vertical lines) for the 6 rats are largest for final values.

(From: Heroux et al, 1956.)

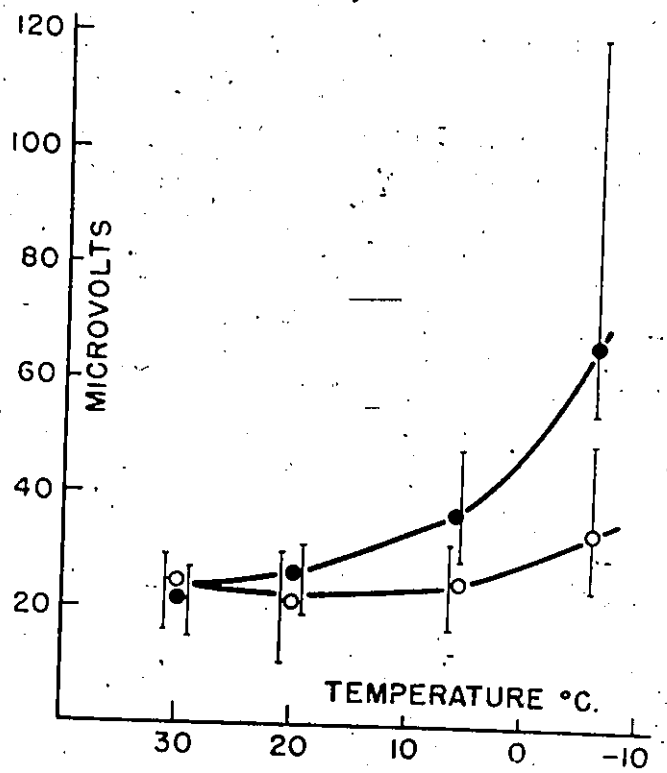

Figure 2. Muscle electrical activity at various temperatures in warm-acclimated (●) and cold-acclimated (o) rats. Vertical bars give range of variation.

(From: Hart et al, 1956)

On the other hand, when shivering is prevented by tubocurarine the warm-acclimated rats fail to maintain body temperature in the cold and they present only a slight rise in oxygen consumption. Carlson and his group (Cottle and Carlson, 1956; Hsieh, Carlson and Gray, 1957) studied this by using rats curarized with tubocurarine chloride and artificially ventilated. As can be seen in Figure 3, they found that the warm-acclimated rats in this condition become hypothermic and present only a small increase in metabolic response to cold.

The mechanism of the shivering thermogenesis localized in skeletal muscle is relatively well understood. The metabolic processes involved in the increase in heat production due to shivering are the same as those involved in the increase in heat production during exercise.

The rate of oxygen and substrate utilization by muscle mitochondria is normally controlled by the supply of ADP rather than Pi. Then the increased utilization of ATP for the muscle contraction of shivering results in an increased availability of ADP which causes accelerated oxidative phosphorylation and electron transport, increased oxygen consumption, increased utilization of substrates, and increased liberation of heat.

Glucose and free fatty acids are the substrates used by the muscle to support the increase in metabolic rate during exposure to cold (for reviews see Himms-Hagen, 1972 a and 1972 b). The increased supply of substrates is under the control of the sympathetic nervous system (SNS) which, through the known metabolic actions of the catecholamines, activates

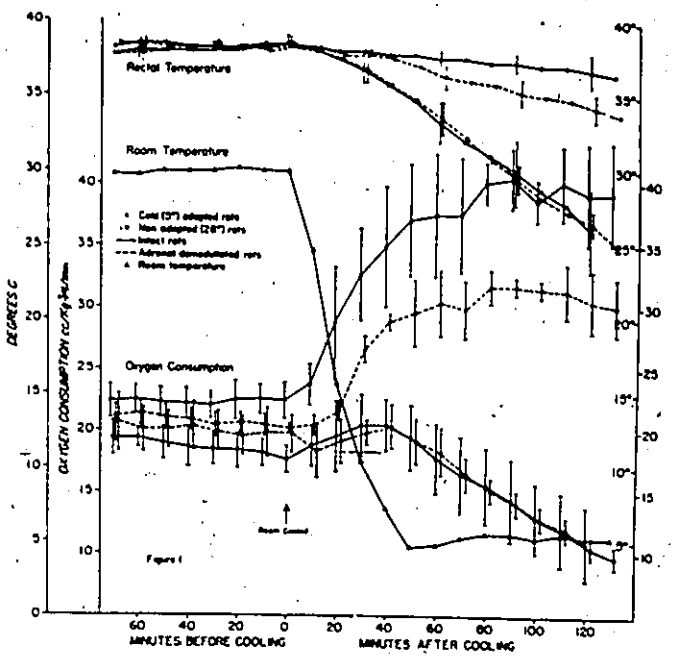

Figure 3. Increase capacity for nonshivering thermogenesis in cold-acclimated rats. Metabolic response of curarized warm-acclimated and cold-acclimated rats to lowered ambient temperature is shown. Mean oxygen consumption and rectal temperature of each group along with ambient temperature is plotted on ordinate; minutes before and after cooling on abscissa. Standard deviations are indicated by vertical lines. The effect of adrenal demedullation on nonshivering thermogenesis in warm-acclimated and of cold-acclimated rats is shown.

(From: Cottle and Carlson,1956.)

glycogenolysis and gluconeogenesis in liver and lipolysis in white adipose tissue.

The SNS is activated in warm-acclimated rats exposed to cold, as indicated by an increase in the urinary excretion of adrenaline and noradrenaline (Leduc, 1961) and their metabolites (for review see Himms-Hagen, 1973), and the liberated catecholamines would be expected to exert a calorigenic effect. In warm-acclimated rats the increase in metabolic rate produced by the secretion of noradrenaline and adrenaline is overshadowed by the much greater increase due to shivering. In order to see the increase in metabolic rate due to catecholamines (nonshivering thermogenesis; see below) it is necessary to suppress shivering with curare-like drugs. But, as was seen in the studies reported by Carlson's group (Fig. 3), the increase in metabolic rate due to nonshivering thermogenesis after shivering has been prevented by tubocurarine is insufficient to maintain body temperature. Himms-Hagen (1967) suggested that the small increase in these cases is due to the use of older rats. However as will be seen, it can be expected that the contribution of nonshivering thermogenesis to the heat production is very small in warm-acclimated rats.

Inactivation of the sympathetic nervous system in some way has been used by several workers in order to study the increase in metabolic rate possibly attributable to catecholamines during exposure to cold. However, this approach creates two new problems. 1) The SNS is essential for the mobilization of substrates during cold-exposure; thus the inactivation of the SNS

can impair the maintenance of the high metabolic rate. 2) When the SNS is inactivated, the mechanisms that reduce heat loss may be impaired (piloerection, peripheral vasocontriction); this would aggravate the hypothermia and complicate the interpretation of results.

The methods for the inactivation of the SNS will be discussed later, when the direct involvement of the SNS in cold-acclimation is reviewed.

Section B: COLD-ACCLIMATION AND NONSHIVERING THERMOGENESIS

It has been long known that cold-acclimated rats do not shiver when exposed to cold, as can be seen in Figure 1 (Héroux et al, 1956). They shiver only if the temperature at which they are exposed is lower than the acclimation temperature (see Fig. 2). In fact, during acclimation to cold the extent of shivering gradually decreases over a period of about 3 weeks, and the contribution of the nonshivering process gradually increases. Two types of evidence support this. One comes from experiments in which shivering has been measured directly by recording the electrical activity of the muscles. Hart et al (1956) showed that after 21 days of continuous cold-exposure shivering, as measured by electromyograms, progressively disappears without any major change in the elevated metabolic rate (see Fig. 4).

The other type of evidence comes from experiments in which shivering has been prevented by tubocurarine. The metabolic response of curarized cold-acclimated rats to lowered ambient

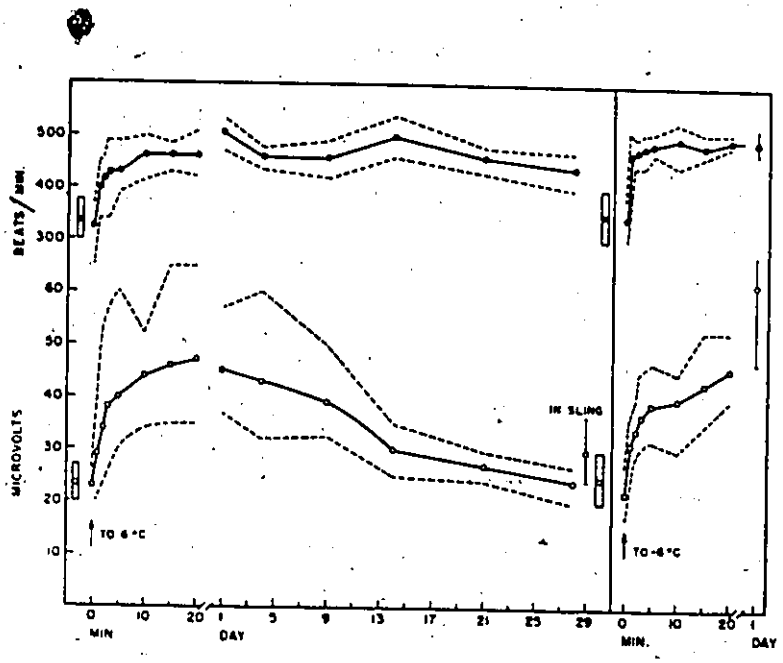

Figure 4. Decrease in shivering (as measured by muscle electrical activity) during acclimation to cold. Muscle electrical activity and heart rate as related to time of exposure to 6°C and -6°C are shown. The solid line in the left-hand part of the diagram shows muscle electrical activity (lower tracing) and heart rate(upper tracing) in rats living at 6°C. The broken lines show the range of variation. The cross-hatched vertical bars at 0 and at 29 days show the muscle electrical activity (lower) and heart rate (upper) in warm-acclimated rats living at 30°C. At 30 days cold-acclimated rats were moved to -6°C (right-hand part of diagram).

From: Hart et al, 1956).

temperature is not impaired and they can maintain their body temperature (Cottle and Carlson, 1956; see Fig. 3).

Nonshivering thermogenesis (NST) has been defined as "a heat-production mechanism liberating chemical energy due to processes, which do not involve muscular contractions" (Jansky, 1973). This broad definition is not precise because it includes the heat production of basal metabolism. Jansky (1973) divided nonshivering thermogenesis into: a) Obligatory or basal NST which serves to maintain the basic energy demands of the homeothermic organism at the temperature of the thermoneutral zone. b) Regulatory NST is the additional heat production which occurs at ambient temperature below the thermoneutral zone.

As these definitions imply that the heat production during the basal metabolism and the heat production during the rapid metabolism in the cold have the same mechanism, it has been decided in this thesis to define nonshivering thermogenesis as the process by which a cold-acclimated animal increases its heat production above the basal metabolic rate by means other than shivering or other muscle movements in the cold.

Obviously, this is also not a precise definition but reflects in great degree the problem to be faced in this study, which is that the mechanism of nonshivering thermogenesis is not understood. Also this definition does not specify the site or sites where this process occurs and therefore it does not clarify if the mechanism is different in the different sites or if it is a common mechanism for all the sites.

Nonshivering thermogenesis has two important characteristics:

1. It is an ADAPTIVE process which occurs only in certain newborn species (rabbit, guinea pig, rat, human), in hibernating mammals (bat, hamster, ground squirrel), and in certain species that have acclimated to cold. It does not occur in these same species when they are adult or not hibernating or not acclimated to cold (see review by Himms-Hagen, 1970).
2. It is also a FACULTATIVE process which does not occur continuously but can be turned on and off in accordance with the requirements of the animal (Depocas, 1960).

The rat and many other species are able to use shivering and nonshivering thermogenesis in response to a cold stress in order to increase their heat production, but the extent to which they will use only one of them or certain proportions of both mechanism will depend on different factors.

a) The time of cold-exposure: As was seen above, when a warm-acclimated rat is exposed to a cold environment it is difficult to assess the contribution of nonshivering thermogenesis to the increase in heat production. It is overshadowed by the large increase due to shivering thermogenesis. As shivering thermogenesis decreases slowly with the time of exposure to cold nonshivering thermogenesis increases slowly.

b) The degree of cold-exposure: This is also important in determining the different proportion of shivering and nonshivering thermogenesis used by the animal under cold stress. As seen in Figure 4 a cold-acclimated rat uses entirely nonshivering thermogenesis at the temperature of acclimation (6°C)

but a more severe cold-exposure (-6°C) provokes shivering. The proportion of heat production due to shivering rises as the temperature is lowered.

c) The age of the animal: Newborn mammals resemble cold-acclimated rats in that they can exhibit nonshivering thermogenesis. Exposure of a newborn mammal to a temperature lower than that to which it is accustomed leads to an increase in oxygen uptake which is not accompanied by shivering. In fact many newborn mammals are unable to shiver and therefore have no alternative mechanism to nonshivering thermogenesis. But during growth at room temperature a newborn animal progressively loses the capacity for nonshivering thermogenesis, so that the adult responds to a cold stress by shivering and not by nonshivering thermogenesis. However, as has already been seen, if a rat is maintained in the cold for a period of 4 weeks shivering thermogenesis progressively disappears and is replaced by nonshivering thermogenesis. Thus the adult rat is able to recall the capacity for making nonshivering thermogenesis that it had just after birth by a prolonged stay in a cold environment. The capacity for developing nonshivering thermogenesis also is age-dependent; older rats cannot become fully cold-acclimated and therefore in the maintenance of the body temperature on cold exposure they may use a greater proportion of shivering thermogenesis than nonshivering thermogenesis.

Section C: ROLE OF THE SYMPATHETIC NERVOUS SYSTEM IN ACCLIMATION TO COLD AND IN NONSHIVERING THERMOGENESIS.

Nonshivering thermogenesis in the cold-acclimated rat is believed to be due to an action of noradrenaline liberated from the nerves of the sympathetic nervous system (SNS). The three principal lines of evidence for this have been reviewed by Himms-Hagen (1967; 1973) and will be summarized here; they are: 1) the activation of the sympathetic nervous system, 2) the mimicking effects of the catecholamines, and 3) the inhibition of the activity of the sympathetic nervous system. It should be emphasized that the SNS is not only involved in the development of nonshivering thermogenesis and in the mechanism of switching on and off of heat production, but is also the main regulator of the necessary substrate mobilization without which no high metabolic rate can be maintained.

1. Activation of the sympathetic nervous system

The sympathetic nervous system is activated during cold-exposure of warm-acclimated rats and cold-acclimated rats as indicated by the large increase in the excretion in the urine of noradrenaline. Leduc (1961) has shown that the excretion of noradrenaline is increased five times in the first day of cold-exposure and is maintained at this level for about 7 days. Thereafter the excretion of noradrenaline declines but remains at elevated levels during several weeks (Fig. 5). The pattern of adrenaline excretion is markedly different. The adrenaline output gradually increases to a maximum in about one week in

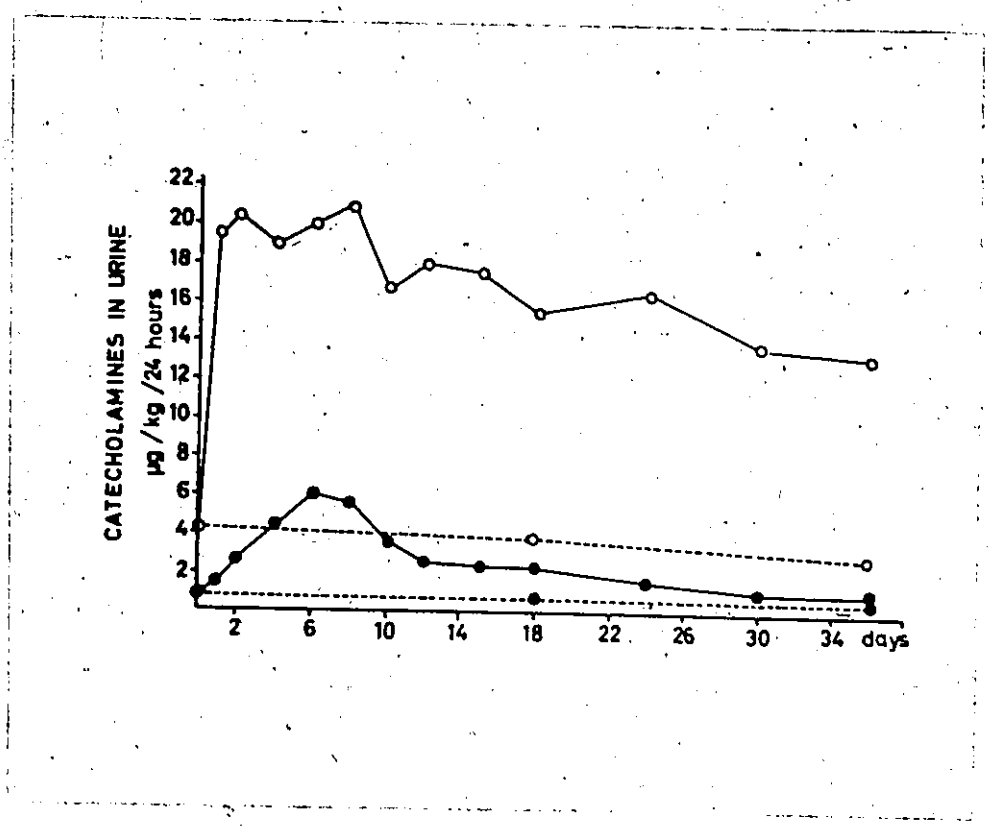

Figure 5. Urinary excretion of adrenaline (●) and noradrenaline (o) in rats (170-180 g) at +3°C (—) and +22°C (---). Each point represents the mean of six individual rats.

(From: Leduc, 1961).

the cold and decreases thereafter more rapidly than that of noradrenaline (Fig. 5). When the rat is cold-acclimated the excretion of adrenaline is only slightly higher than that of the control group of warm-acclimated rats. Leduc (1961) also showed that the excretion of catecholamines is proportional to the temperature of exposure and is greater in younger animals and that when rats are removed from the cold the catecholamine excretion values return to normal within one or two days at room temperature.

In order to determine if the increase in urinary levels of noradrenaline seen in the cold reflects an increase in secretion or a decrease in metabolic degradation, Shum et al (1969) studied the influence of cold-exposure on the secretion and metabolic degradation of adrenaline and noradrenaline. They showed that together with the increase in noradrenaline secretion there was an increase also in its major metabolites, normetanephrine (NM) and 3-methoxy-4-hydroxyphenylglycol (MHPG), during cold-exposure and during cold-acclimation. MHPG is the major metabolite of noradrenaline in the rat and some authors have proposed the use of MHPG excretion as an index of noradrenaline biosynthesis (Iversen, 1967). Therefore, changes in MHPG excretion can be interpreted to reflect alterations in noradrenaline synthesis. Thus, in cold-exposure and during cold-acclimation there is an increase in the synthesis of the catecholamines, particularly of noradrenaline.

2. Mimicking effects of the catecholamines

A major line of evidence for the participation of the catecholamines in nonshivering thermogenesis is the marked calorigenic action of these compounds, an action which is enhanced in cold-acclimated rats just as is the capacity for nonshivering thermogenesis.

Hsieh and Carlson (1957) provided the first demonstration that the calorigenic effect of noradrenaline was greatly enhanced in cold-acclimated rats. Figure 6 illustrates the marked difference between the response of a warm-acclimated rat and a cold-acclimated rat to the infusion of noradrenaline (Himms-Hagen, 1970).

Intravenous infusion of adrenaline induces a similar response (Fig. 7). This result is in disagreement with the previous report of Hsieh and Carlson (1957). However, this can be explained on the basis of the different route of administration used in both cases: intramuscular administration by Hsieh and Carlson (1957), intravenous infusion by Himms-Hagen (1969). Thus it is now apparent that cold-acclimated rats acquire an enhanced calorigenic response to adrenaline as well as to noradrenaline. Therefore it cannot be excluded that adrenaline secreted from the adrenal medulla also contributes to nonshivering thermogenesis.

As can be seen in Figures 6 and 7, the enhanced calorigenic response of cold-acclimated rats to catecholamines is turned off as soon as the infusion of the catecholamine is stopped.

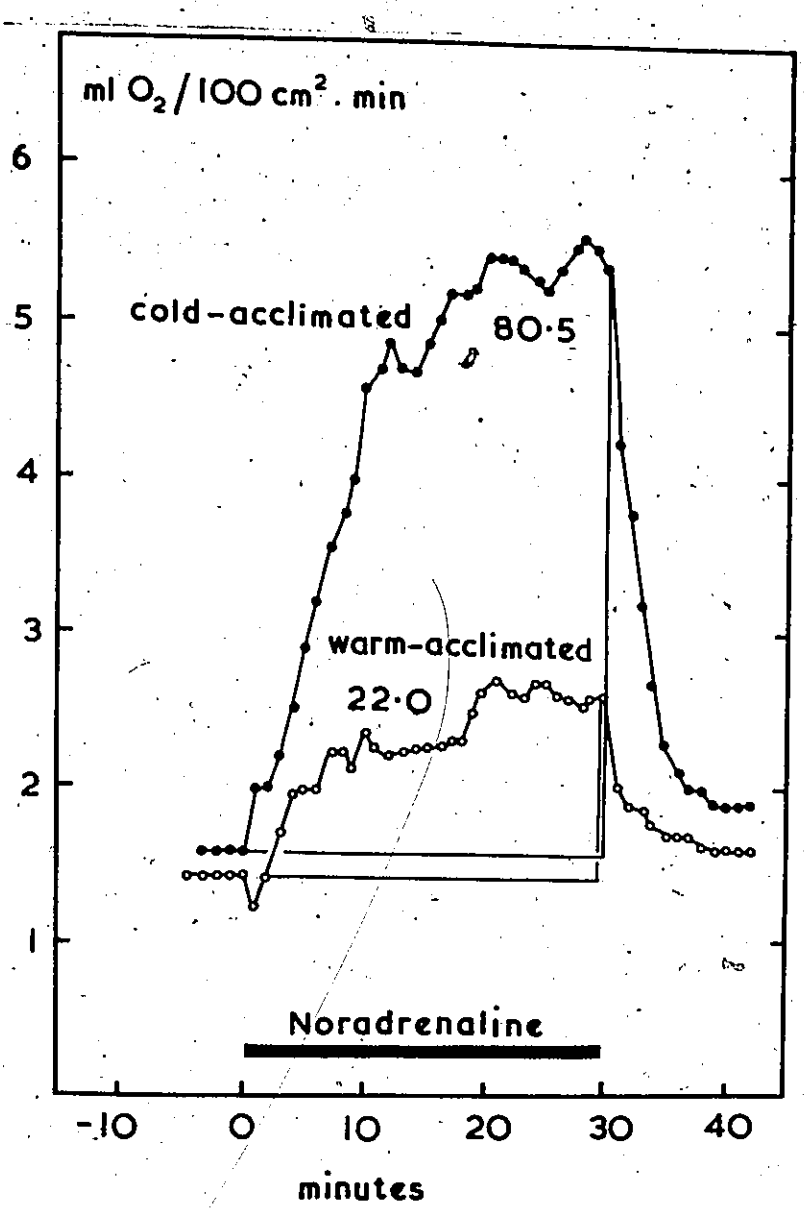

Figure 6.

Enhancement of the calorigenic response to noradrenaline by cold-acclimation. Oxygen uptake of a warm- and a cold-acclimated rat during infusion of noradrenaline is shown. Noradrenaline was infused intravenously, 0.5 µg/100 cm^2.minute, from 0 to 30 minutes. The rats were lightly anaesthetized with sodium pentobarbital. The warm-acclimated rat weighed 478 g and the cold-acclimated rat weighed 373 g; they had lived at room temperature (25°-28°C) and in the cold (4°C) respectively for 13 weeks. The values of 80.5 and 22.0 on the graph are obtained from the area under the curve during the 30 minutes of infusion of noradrenaline and they represent the total increase in oxygen uptake in ml O_2/100 cm^2.in 30 minutes. The increases shown are typical of rats kept under these conditions.

(From: Himms-Hagen, 1970)

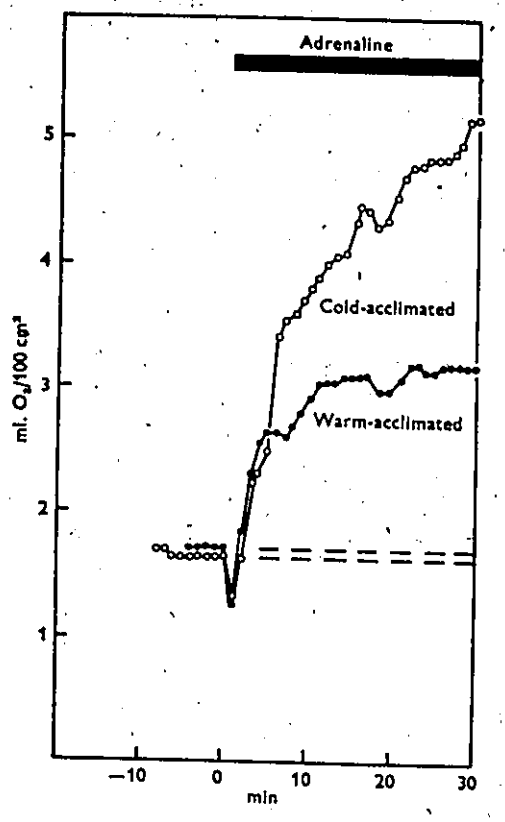

Figure 7. Enhancement of the calorigenic response to adrenaline by cold-acclimation. Oxygen uptake of a warm- and a cold-acclimated rat during infusion of adrenaline is shown. Adrenaline was infused intravenously, 0.5 µg/100 cm^2. minute from 0 to 30 minutes. The rats were lightly anaesthetized with sodium pentobarbital. The warm-acclimated rat weighed 415 g, the cold-acclimated rat weighed 331 g; they had lived at room temperature (25°-28°C) or at 4°C for 9 weeks.

(From: Himms-Hagen, 1969).

Therefore the calorigenic action of catecholamines mimicks relatively well one of the characteristics of NST; the facultative nature of its occurrence, i.e., its rapid switching on and off.

The enhancement of the calorigenic response to noradrenaline develops slowly during the first 4 weeks of exposure to cold (Depocas, 1960; Himms-Hagen, 1969; Bartunkova et al, 1971; see Fig. 8). During this period the extent to which the rats shiver while in the cold decreases slowly, reaching a minimum at about the same time as the calorigenic response to the catecholamine reaches a maximum which parallels the development of nonshivering thermogenesis (see Fig. 4).

The enhancement of the calorigenic response is not due to an increased sensitivity to noradrenaline, but to an increase in the maximum attainable response (Himms-Hagen et al, 1972). The fact that there is not a shift of the dose response curve (see Fig. 18) permits the conclusion that the enhancement is not due to an increase in sensitivity to noradrenaline.

The magnitude of the calorigenic action of noradrenaline progressively disappears during cold-deacclimation (Bartunkova et al, 1971; see Fig. 9). Also it is known that the magnitude of the calorigenic action of noradrenaline is inversely proportional to the acclimation temperature (Jansky et al, 1967; see Fig. 10).

Not only is the sympathetic nervous system involved in switching on and off of nonshivering thermogenesis but the increased secretion of noradrenaline also plays a role in the

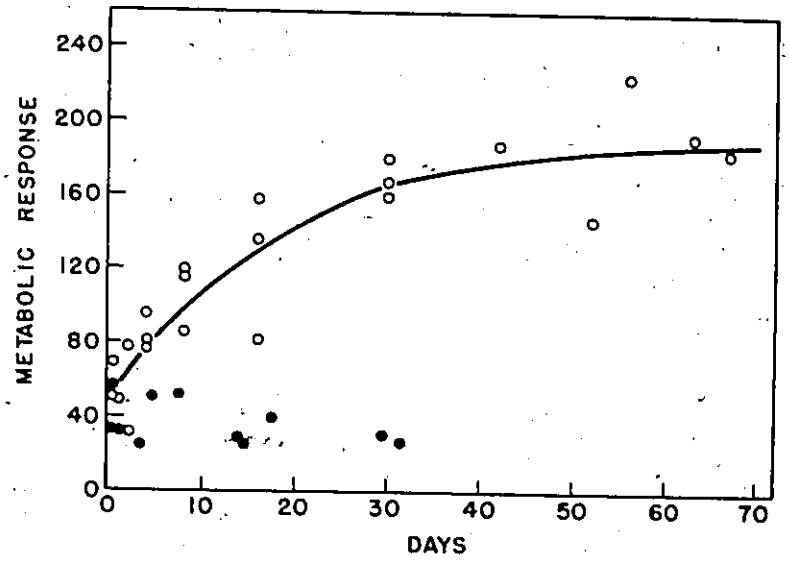

Figure 8. Development of the enhanced calorigenic response to noradrenaline during acclimation to cold. Metabolic response to intravenously infused L-noradrenaline bitartrate at the level of 1 µg free base per minute per rat in rats previously maintained at 30°C (●) and 6°C (o) is shown. The metabolic response is given in square centimeters and corresponds to the area under the curve of oxygen consumption vs.time during noradrenaline infusion (100 minutes) minus the area corresponding to the initial oxygen consumption in the same period of time. The average increase in milliliter O_2 consumed per minute for each rat, during infusion of noradrenaline, can be obtained by dividing the metabolic response units by 20.

(From: Depocas,1960).

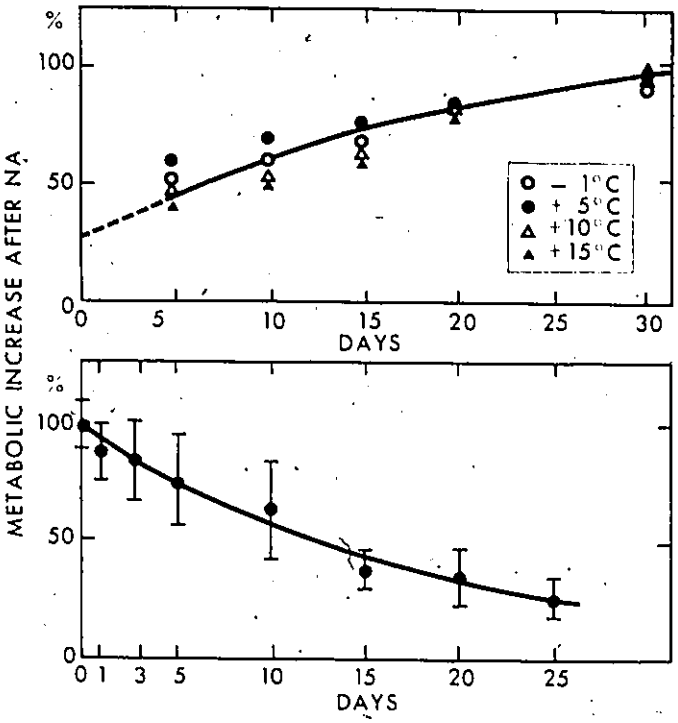

Figure 9 A. Development of the enhanced calorigenic response to noradrenaline during acclimation to cold. Metabolic response to intramuscular injections of noradrenaline (0.4 mg/Kg) are expressed as percentage of the value obtained after 40 days of acclimation to different temperatures.

Figure 9 B. Decrease of the calorigenic response to noradrenaline in cold-acclimated rats at 5°C exposed to 25°C. Metabolic response to intramuscular injections of noradrenaline (0.2 mg/Kg) are expressed as percentage of the initial metabolic response of cold-acclimated rats.

(From: Bartunkova et al, 1971)

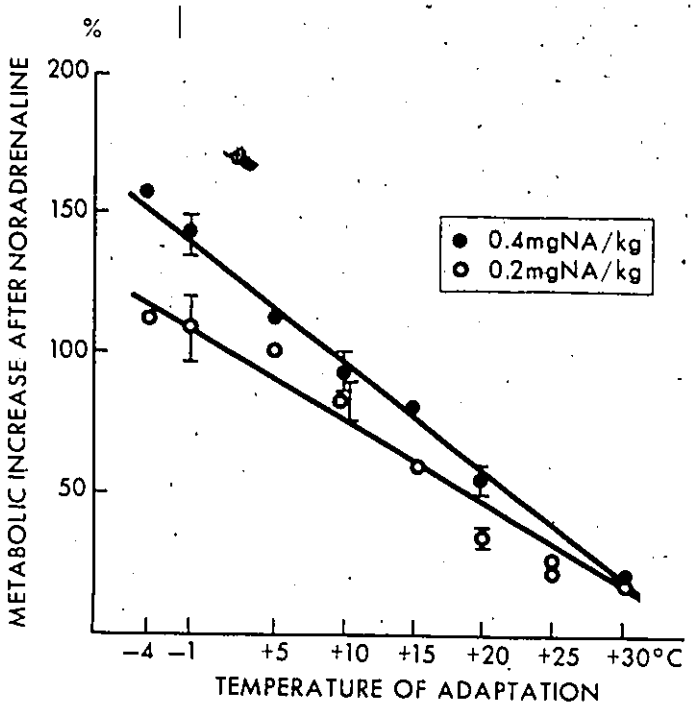

Figure 10. Relationship between maximun metabolic response in rats after intramuscular administration of noradrenaline expressed as a percentage of resting values,and the temperature to which the rats were acclimated for three weeks. Perpendicular lines represent SD.

(From: Jansky et al, 1967)

development of adaptation to cold. Prolonged treatment of rats with noradrenaline produces an adaptive state similar to that of cold-acclimation in that the calorigenic response to noradrenaline is enhanced and the resistance to cold is increased (LeBlanc and Pouliot, 1964).

In summary, the sympathetic nervous system not only operates the switching on and off of nonshivering thermogenesis in the cold-acclimated rat but also participates in the development by the rat living in the cold of the capacity for nonshivering thermogenesis. The measurement of the calorigenic action of noradrenaline can be very useful for the measurement of nonshivering thermogenesis because it mimicks relatively well its two fundamental characteristics: the facultative process of its rapid switching on and off, and the adaptive process in the increase for the capacity for making nonshivering thermogenesis during acclimation to cold.

3. Inhibition of the activity of the sympathetic nervous system

It is possible to inhibit part of the function of the sympathetic nervous system (SNS) by surgical, immunological or pharmacological means. In order to see the role of adrenaline secreted by the adrenal medulla in cold-acclimation, rats can be demedullated by surgical removal of the adrenal medulla.

Young rats lacking an adrenal medulla do not maintain their body temperature and do not survive very long when exposed to cold (see Himms-Hagen, 1973). On the other hand adrenodemedullated adult rats are able to survive in the cold and their

ability to maintain body temperature in the cold without appreciable shivering indicates that they have become cold acclimated (Schönbaum et al, 1963). However, if rats already cold-acclimated are demedullated, and then reexposed to cold, they show a much smaller increase in metabolic rate and become somewhat hypothermic as can be seen in Figure 3 (Cottle and Carlson, 1956).

Thus the adrenal medulla, and consequently adrenaline, seems to play an important role in the heat production in young rats and in cold-acclimated rats; but in older rats the sympathetic nervous system apparently compensates for the removal of the adrenal medulla by increasing the role of some of its other constitutive parts in order to maintain survival of the older animals (see review by Himms-Hagen, 1973).

In order to study an animal possessing only the adrenal medulla but lacking the rest of the SNS several techniques can be used: surgical, immunological or pharmacological. However at the moment these techniques are not entirely satisfactory due to the lack of completeness or lack of specificity. It is often necessary to use a combination of them in order to show the involvement of the SNS in cold-acclimation.

Immunosympathectomy. This technique results in the lack of adrenergic nerve endings in some tissues as a consequence of the repeated administration to fetal and/or newborn animals of antiserum to nerve growth factor (see Levi-Montalcini and Angeletti, 1966). Immunosympathectomy does not alter the ability of rats to survive in the cold (Berti et al, 1965) but if the

adrenal medulla is also removed, then the rats became hypothermic in the cold. Thus the adrenal medulla seems to have the capacity to play a role in the calorigenic response to cold. Schönbaum et al (1966) have shown that immunosympathectomized and adrenodemedullated older rats can become cold-acclimated, but this can be due to the remaining activity of the SNS not affected by the treatment (Himms-Hagen, 1973). On the other hand, immunosympathectomy prevents the calorigenic response to cold-exposure in newborn rats and causes hypothermia and death (Wekstein, 1964); these rats cannot shiver and their adrenal medulla is still immature so they have no alternative calorigenic mechanism to the nonshivering thermogenesis mediated by the sympathetic nerve endings.

Pharmacological agents. Reserpine, adrenergic blocking agents, ganglionic blocking agents, beta-adrenergic blocking agents, and inhibitors of the synthesis of catecholamines have been widely used (for review see Himms-Hagen, 1973) with variable results.

Administration of the ganglionic blocking agent hexamethonium has been one of the most successful. In fact, Hsieh et al (1957) found that curarized cold-acclimated rats exposed to cold and treated with hexamethonium cannot show the rise in oxygen uptake induced by exposure to cold (see Fig. 11) and that this drug can reverse the increase in oxygen uptake if the administration is made after the increase has already occurred (see Fig. 12). As can also be seen in Figure 12 noradrenaline can reverse the inhibitory effect of hexamethonium whereas

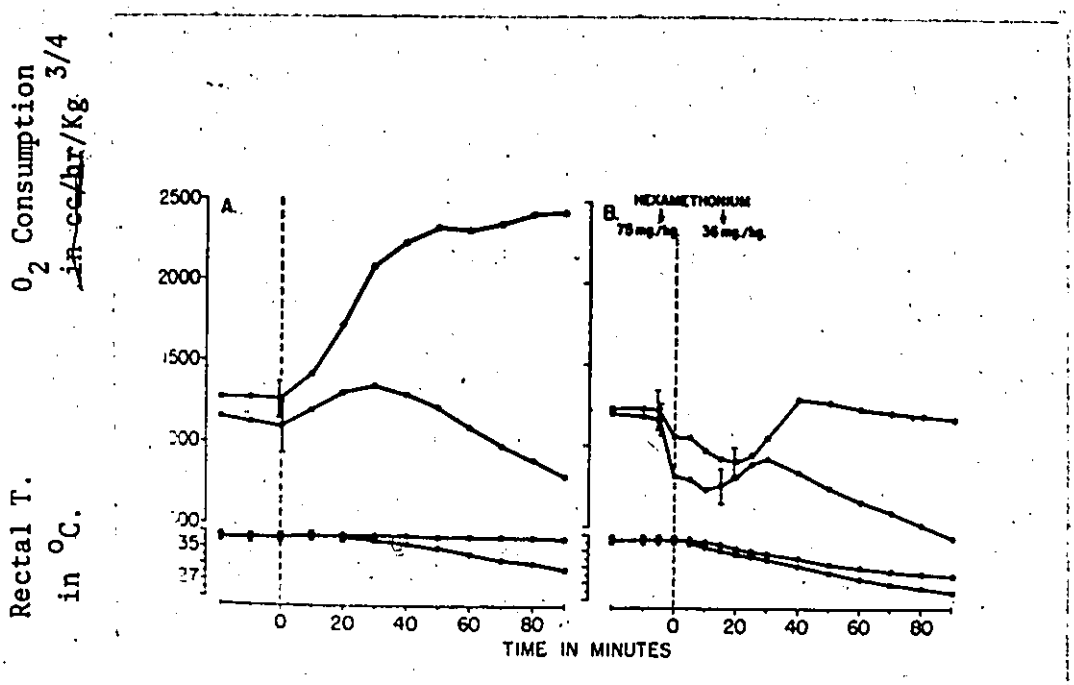

Figure 11. Oxygen consumption and rectal temperature of curarized, cold-acclimated rats (o) and warm-acclimated rats (o) receiving hexamethonium. The room was cooled to 5^{o}C at zero time. Hexamethonium was injected intravenously at the points indicated by the arrows.

(From: Hsieh et al, 1957).

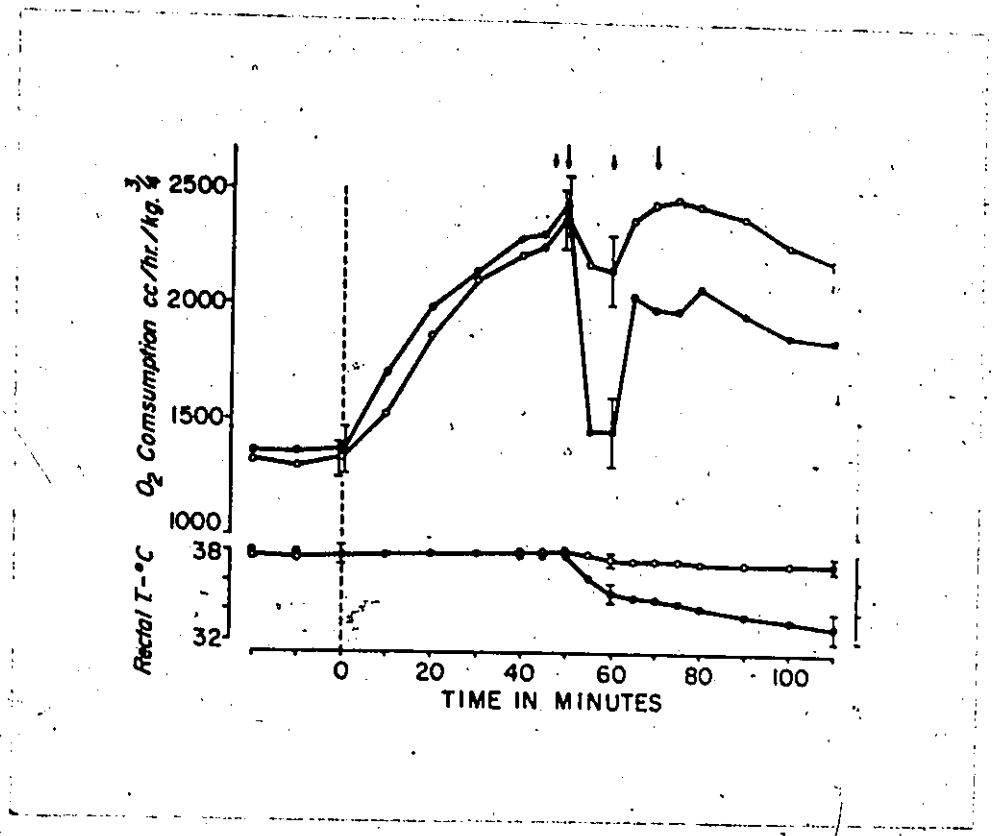

Figure 12. Oxygen consumption and rectal temperature of curarized, cold-acclimated rats receiving hexamethonium and adrenaline or noradrenaline. The room was cooled to $5^{o}C \pm 1^{o}C$ at zero time. Hexamethonium was injected intravenously at the points indicated by the long arrows. At the points indicated by the short arrows, adrenaline (0.2 mg/Kg) was injected intramuscularly to the group represented by solid dots and l-noradrenaline in the same dose given to the group represented by the open circles.

(From: Hsieh et al, 1957).

adrenaline is less effective. However, the catecholamines were administrated by intramuscular injection and it is not possible to draw any definitive conclusion regarding the efficiency of those hormones to reverse hexamethonium effects. Himms-Hagen (1969) has shown that adrenaline and noradrenaline administered by intravenous injection are equally efficient in the mimicking of nonshivering thermogenesis.

In summary the use of these methods or a combination of some of them demonstrates that the sympathetic nervous system is directly involved in the switching on and off and in the development of nonshivering thermogenesis. However, it is very difficult to draw more precise conclusions in view of the fact that the extent to which the two major divisions of the sympathetic nervous system (adrenal medulla and sympathetic nerve endings) participate in the reactions involved in survival in the cold is still obscure. The sympathetic nervous system is a highly efficient emergency mechanism in that although the functions of its parts are normally well integrated, a small part can nevertheless take over the function of the other parts when necessary (Himms-Hagen, 1973).

Section D: MUSCLE AS A MAJOR SITE OF NONSHIVERING THERMOGENESIS

There has been a long controversy about the site of heat production during nonshivering thermogenesis. Originally, visceral organs were considered as the main source of heat under these conditions, since it was found by many authors that the temperature of the liver or other internal organs increased

after exposure of animals to cold. However, temperature changes in the various organs do not give suitable information about thermogenesis in those organs since they are influenced by the blood flow and do not give any information about the amount of heat produced under these conditions.

Therefore other experimental approaches have been used which will be presented in the following discussion of the relative participation of each individual organ in nonshivering thermogenesis.

Skeletal Muscle

Depocas (1958) was the first who clarified the problem of the major site of heat production in the cold-acclimated rat with an experiment in which cold-exposed eviscerated cold-acclimated rats showed a large increase in oxygen uptake (which was only slightly lower than that of the sham-operated cold-acclimated control group) as can be seen in Figure 13. As the evisceration technique used prevented blood flow through the stomach, intestines, spleen, pancreas and liver, Depocas thus proved that these viscera are not required for the switching on of nonshivering thermogenesis and that the extravisceral tissues are quantitatively more important sites for nonshivering thermogenesis.

In order to estimate the effect of evisceration on the response of cold-acclimated rats to noradrenaline infusion Depocas (1960 a) performed a similar experiment in which cold-exposure was replaced by noradrenaline infusion. As can be seen in Figure 14, the eviscerated cold-acclimated rat during the

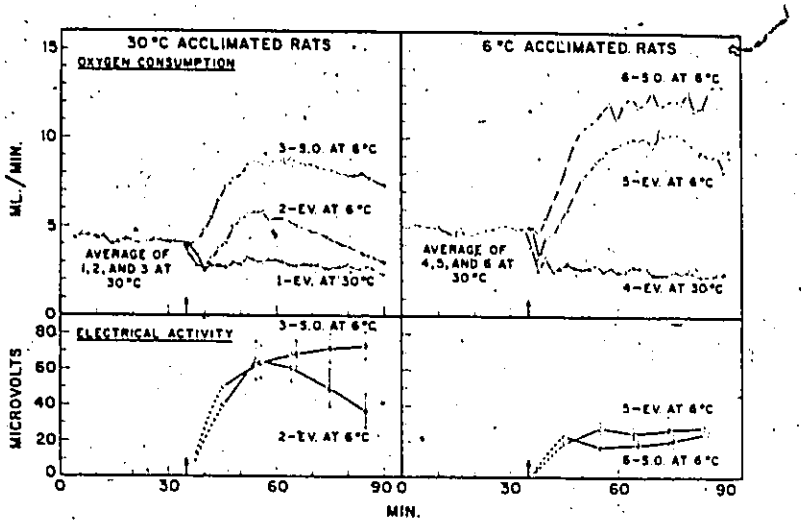

Figure 13. Persistence of nonshivering thermogenesis in functionally eviscerated rats. Average 0_2 consumption and muscle electrical activity of barbital-anaesthetized 30° and 6° acclimated rats at 30°C (average 0_2 consumption of all similarly acclimated rats given for interval 0-35 minutes), at 30° C after evisceration (groups 1 and 4), at 6°C after evisceration (groups 2 and 5), and at 6°C after sham-operation (groups 3 and 6) are shown. Arrow indicates time of evisceration or transfer to 6°C or both. Al groups consisted of five animals except group 3 in which there were six. Evisceration consisted of tying off the rectal colon, coeliac and superior mesenteric arteries and the portal vein; this procedure effectively prevents blood flow through intestine and liver.

(From: Depocas, 1958)

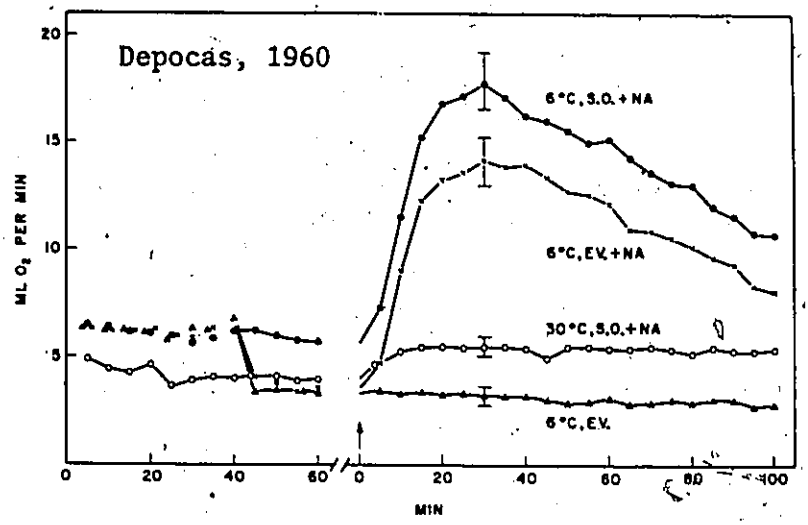

Figure 14. Persistence of an enhanced calorigenic response to noradrenaline in functionally eviscerated cold-acclimated rats. Average oxygen consumption of barbital anaesthetized cold-acclimated rats (three rats per group), functionally eviscerated and infused with 0.1% sodium bisulphite in saline (6°C, EV.), functionally eviscerated and infused with noradrenaline (in 0.1% sodium bisulphite in saline) at a dose of 1 µg per minute (6°C, EV.+ NA), sham-operated and infused with noradrenaline at the same dose level (6°C, S.O.+ NA). The average oxygen consumption of two warm-acclimated rats, sham-operated and infused with noradrenaline, is also given. Arrow indicates start of infusion wich lasted 100 minutes. Vertical bars indicate total range of variation.

(From: Depocas, 1960)

infusion of noradrenaline has a similar metabolic response to the cold-acclimated sham-operated rat. These observations also provide further evidence for the suggested role of noradrenaline as the mediator of nonshivering thermogenesis because it can mimick very well the effect of cold in the cold-acclimated rat (compare Fig. 14 with Fig. 13).

In view of this evidence Depocas (1960 a) suggested that skeletal muscle should be considered as a possible site for nonshivering thermogenesis in the cold-acclimated rat.

In order to obtain more direct evidence for the participation of muscle in nonshivering thermogenesis Jansky and Hart (1963) elaborated a method which permitted the measurement "in situ" of oxygen consumption of muscles of the hind leg of cold-acclimated rats. The oxygen consumption was calculated from arterio-venous differences in blood oxygen content and from blood flow rates after noradrenaline infusion or exposure to cold. Figure 15 shows that the oxygen uptake of leg muscle was more than doubled during infusion of noradrenaline or during exposure to cold as was also the total metabolism of the rat. It should be noted that the authors point out that the increased oxygen uptake in leg muscles produced by noradrenaline or cold-exposure was due entirely to the increase in arterio-venous oxygen differences and not to an increase in blood flow. This suggests that muscle can increase its capacity for taking up oxygen out of the blood without an increase in oxygen supply. Although these data point to the significant role of the skeletal muscle in nonshivering thermogenesis, these observations do not

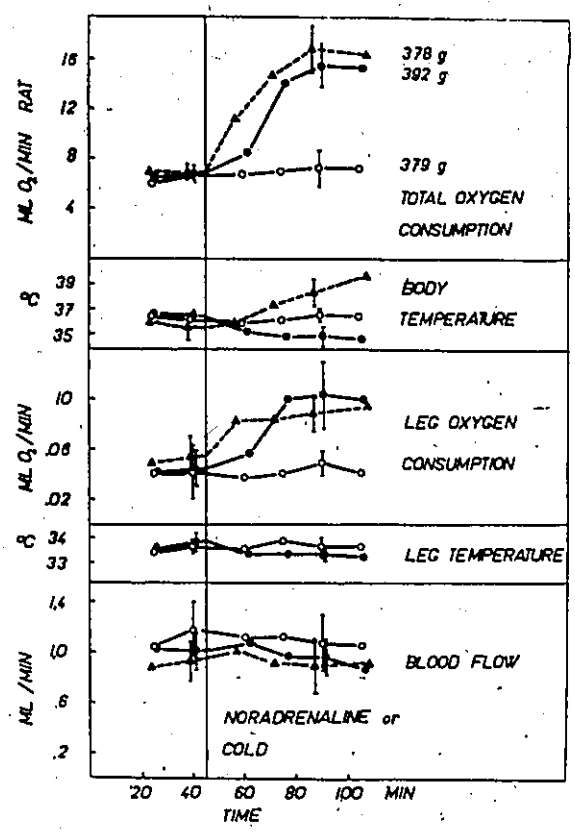

Figure 15. Occurrence of nonshivering thermogenesis and of a calorigenic response to noradrenaline in leg muscle of cold-acclimated rats. Comparison of responses in the leg and in the whole rat before and during noradrenaline infusion at 0.8 µg/min (▲) or exposure to 10°C (●) Vertical line at 45 minutes shows start of noradrenaline or cold. Untreated controls (o) are also indicated. Vertical lines denote standard deviation. Results not corrected for change in body temperature during tests.

(From: Jansky and Hart, 1963)

allow an estimation of the quantitative significance of the metabolism of the whole muscle mass for nonshivering thermogenesis.

In order to cope with this problem an indirect method was developed by Jansky. It is based on the assumption that the total cytochrome oxidase activity can be an indicator of the maximal metabolic capabilities of the whole animal as well as of individual organs (Jansky, 1961, 1963). This method, however, showed that the metabolic capacity of the muscles is not sufficient to cover the metabolic increase due to shivering and nonshivering thermogenesis in cold-adapted rats. It was estimated that probably only 50-70% of nonshivering thermogenesis* can have its origin in the muscles (Jansky, 1966).

In 1968 Jansky and Hart measured the cardiac output and the organ blood flow in warm-acclimated and cold-acclimated rats exposed to cold from which they were able to calculate the maximum oxygen consumption that would result if all the oxygen were removed from the blood passing through the organ. On the basis of such calculations it was estimated that the metabolic contribution of skeletal muscle to nonshivering thermogenesis is of the order of 50% of the total. It is clear that this method does not provide any information about the real oxygen consumption of an individual tissue or its participation in the total nonshivering thermogenesis, because each tissue consumes

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* The indicated percentage corresponds to the definition of nonshivering thermogenesis of Jansky (1973), which includes the basal metabolic rate.

an unknown proportion of the oxygen supplied to it. However, as it will be seen, this method is very useful to eliminate as important sites of nonshivering thermogenesis certain tissues which do not receive enough oxygen.

It should be noted that Jansky (1966) arrived at a good agreement between the indirect method of cytochrome oxidase activity of different tissues and the method from blood flow measurements (Jansky and Hart, 1968). However, it is possible to obtain values of cytochrome oxidase much higher (Himms-Hagen et al, 1972) than those obtained by Jansky.

Therefore none of the methods used can give the real quantitative contribution of an organ to the total nonshivering thermogenesis. To quote Jansky (1971) "The best and ideal technique would be one based on organ blood flows and on extraction of oxygen from the blood. However, this represents a great obstacle in small animals where cannulation of small vessels under physiological conditions is practically impossible".

Liver

As the dominant role of skeletal muscle and the minimal participation of visceral organs in nonshivering thermogenesis has been already demonstrated by the evisceration experiment of Depocas (1958) Jansky et al (1964) attempted to determine the exact significance of the liver in nonshivering thermogenesis. As measurements of liver oxygen consumption of small mammals under in vivo conditions is technically impossible owing to the complicated vascularisation of this organ they performed experiments on isolated livers perfused with undiluted rat blood,

before and after injection of a high concentration of noradrenaline into the circulation. Under these conditions, no change in liver metabolism after noradrenaline was observed. Based on indirect methods such as cytochrome oxidase activity and blood flow changes it has been estimated that liver metabolic contribution to nonshivering thermogenesis could not exceed 25% (Jansky, 1971,1973).

Earlier observations of Kawahata and Carlson (1959) indicated that liver contribution to nonshivering thermogenesis is very low. They showed that the blood flow through the liver increases only 10% in curarized cold-acclimated rats exposed to cold, and that there was no difference in the oxygen tension between livers of warm-acclimated and cold-acclimated animals.

Kidneys

Jansky and Hart (1963) also measured in situ the arteriovenous differences in blood oxygen content and blood flow of kidney of curarized cold-acclimated rats exposed to cold or injected with noradrenaline. No metabolic increase was observed under these conditions although it was found that the kidney blood flow increases during cold-exposure (Jansky and Hart, 1968).

As measured by the cytochrome oxidase technique (Jansky, 1966) the kidney could account for only 5% of the total metabolic response. Therefore, Jansky (1971,1973) concluded that the kidneys do not participate in nonshivering thermogenesis.

Intestine

Intestine was also eliminated as an important site for nonshivering thermogenesis by the evisceration experiment (Depocas, 1958). However, from blood flow measurements (Jansky and Hart, 1968) this organ appeared to be a potential heat producer. Therefore, Jansky (1971) carried out experiments in which the participation of intestine in nonshivering thermogenesis was measured directly by blood flow measurements and arterio-venous differences in oxygen tension between the aorta and the portal vein in cold-acclimated rats, before and after exposure to cold. No change was observed in the arterio-venous differences in oxygen content, but as the blood flow increased by 55% it was calculated that the total oxygen uptake in the intestine increase by 64%, which represent less than 10% of nonshivering thermogenesis.

Skin

Jansky and Hart (1963) showed that the separation of the skin from the leg had no significant effect on leg oxygen consumption during noradrenaline infusion "in situ". Moreover it has been shown that cold-exposure of cold-acclimated rats does not change the blood flow to the skin (Jansky and Hart, 1969). By estimation of the metabolic capacity of the skin from total cytochrome oxidase activity, Jansky (1966) determined that skin participation in nonshivering thermogenesis is not more than 6%. Thus it is unlikely that the skin has a significant role in nonshivering thermogenesis.

Brown adipose tissue

Much attention has also been devoted to the thermogenic significance of brown adipose tissue. The properties, distribution and heat production of this tissue have been reviewed by Smith and Horwitz (1969) and Himms-Hagen (1970), and will only be summarized here in order to ascertain how brown adipose tissue could contribute to nonshivering thermogenesis.

When warm-acclimated rats are exposed to cold, the brown adipose tissue hypertrophies, It is known that the new cells are derived from endothelial cells (Cameron and Smith, 1964), just as the normal brown adipose tissue cells are during differentiation before birth (Barnard, 1969). Therefore during adaptation to cold the heat producing capacity of this tissue can be assumed to become greater both because it has more cells and because the cells have more mitochondria which contain more cristae and respiratory enzymes (Skala et al, 1970). More recently, Lindgren and Barnard (1972) have reported that an absolute increase occurs in the area of inner membrane, whereas the outer membrane of the brown adipose tissue mitochondria is not structurally affected by cold-acclimation.

The brown adipose tissue, and particularly the interscapular brown adipose tissue, is the warmest region of the body when nonshivering thermogenesis occurs and under those circumstances the oxygen consumption and the blood flow are increased (Himms-Hagen, 1970). However in spite of the increase in the heat-producing capacity of the brown adipose tissue during adaptation

to cold the enlarged tissue still represents only 1% of the total body mass and this is in disfavour of an important contribution of this tissue to nonshivering thermogenesis. In fact, several estimates of the contribution of brown adipose tissue to total oxygen uptake in an adapted animal to cold during infusion of noradrenaline or during exposure to cold give values between 6 and 12% of the total oxygen uptake (Himms-Hagen, 1972). However other species such as the newborn rabbit have a much larger amount of brown adipose tissue relative to their total body mass (about 5% in the newborn rabbit) and this could contribute to about 80% of the total heat production during cold-exposure or during infusion of noradrenaline (Hull and Segal, 1965). In fact, the surgical removal of the interscapular brown adipose tissue (which represent about 30% of the total brown adipose tissue) in the newborn rabbit results in an immediate and important loss in the calorigenic response to cold-exposure and noradrenaline. But the same operation has no immediate effect on the cold acclimated rat, as measured by the calorigenic response to adrenaline or to noradrenaline (Himms-Hagen, 1969; see Figs. 16 and 17).

In summary, brown adipose tissue has not a quantitative importance in its contribution to the nonshivering thermogenesis in the cold-acclimated rat. In the next section will be discussed the qualitative importance of the metabolism of this tissue in nonshivering thermogenesis.

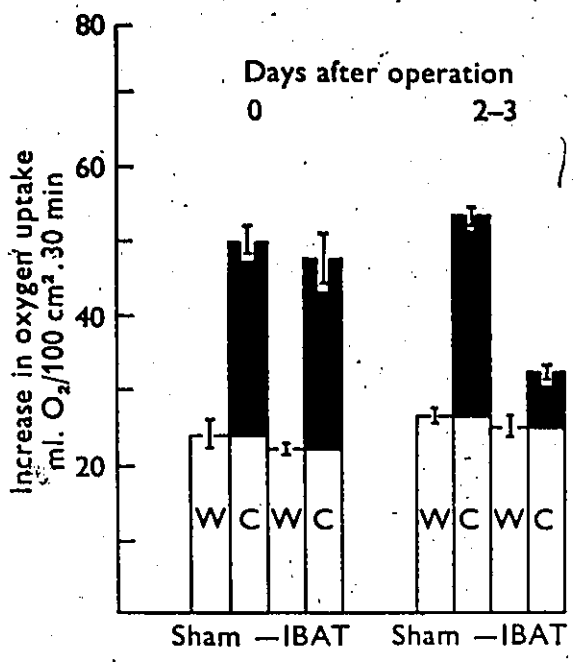


Figure 16.

Delayed effect of removal of the interscapular brown adipose tissue on the enhanced calorigenic response to adrenaline in cold-acclimated rats. Each bar represents the mean of values from ten rats with the S.E. indicated by the vertical line. The mean weight of the cold-acclimated rats at the time they were removed from the cold was 265 g; they had lived in the cold for only 1-2 weeks. The mean weights of the warm-acclimated rats at this time was 309 g. Warm-acclimated rats are indicated by W; cold-acclimated rats are indicated by C. The portion of the bars for cold-acclimated rats that is coloured black is the difference in response between cold-acclimated rats and the corresponding warm-acclimated rats and represents the enhancement of the calorigenic response to adrenaline caused by acclimation to cold. Values obtained immediately after the operation are shown on the left of the diagram (day 0);those obtained 2-3 days later are shown on the right. Sham-operated controls are labelled Sham;rats with their interscapular brown adipose tissue removed are labelled -IBAT.

(From: Himms-Hagen, 1969)

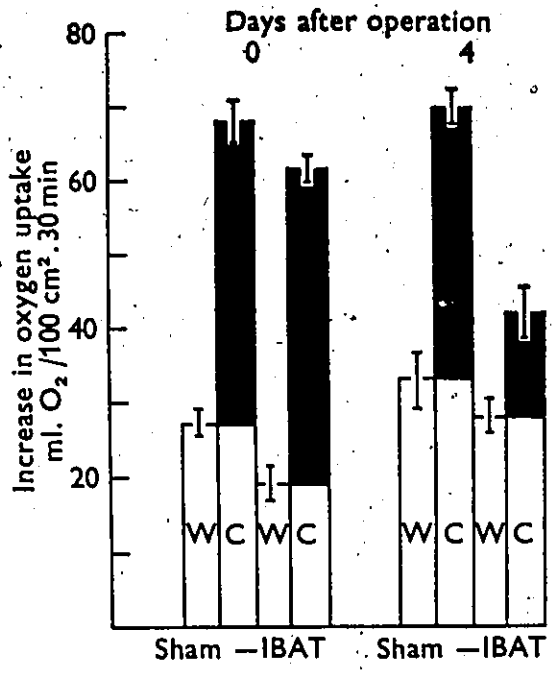


Figure 17. Delayed effect of removal of the interscapular brown adipose tissue on the enhanced calorigenic response to noradrenaline in cold-acclimated rats. Each bar represents the mean of values from six rats with the S.E. indicated by the vertical line. The mean weight of the cold-acclimated rats at the time they were removed from the cold was 332 g: they had lived in the cold for 6-11 weeks. The mean weight of the warm-acclimated rats at this time was 432 g. Other symbols are the same as in Figure 16.

(From: Himms-Hagen, 1969)

Other tissues

Jansky (1966) estimating metabolic capacity from total cytochrome oxidase activity attributed to heart, lung, brain, testes and spleen a very low metabolic capacity. The estimation based on blood flow and cardiac output to various organs (Jansky and Hart, 1968) showed that small tissues like heart, diaphragm, lung, spleen, thymus, pancreas, white fat, testes and adrenals cannot be important sites of nonshivering thermogenesis. No attempt has been made to evaluate directly the role of small body organs. However, because of their small mass their thermogenetic importance is probably not significant.

In summary, there are several lines of evidence that skeletal muscle is the major site of nonshivering thermogenesis. From the reports presented its participation can be evaluated as 50-70% of the total nonshivering thermogenesis, the rest being distributed between various tissues in which the liver, the intestine and brown adipose tissue are the most important sites.

Thus, as skeletal muscle is the major site of nonshivering thermogenesis in the rat, this tissue has been selected for the experimental work described in this thesis in which the aim is to elucidate some of the biochemical mechanisms involved in nonshivering thermogenesis.

Section E: THE SPECIAL ROLE OF BROWN ADIPOSE TISSUE IN NONSHIVERING THERMOGENESIS

Among mammals brown adipose tissue has been found in appreciable quantities in certain newborn, cold-acclimated and hibernating animals. It is known that the amount of the brown adipose tissue decreases with the age of the animal, when the animal is not hibernating or when it is not adapted to cold. Smith and Horwitz (1969) presented a comprehensive review of the distribution, structure and metabolism of brown adipose tissue.

The distribution of the brown adipose tissue is diffuse in the adult rat; the areas where brown adipose tissue generally occurs are:

a) a pair of brown fat pads located deep in the middorsal superior cervical region,
b) the interscapular region with extensions ventrolaterally which engulf the vessels of the axillary region,
c) the thorax where this tissue overlays the aorta and azygous vein as well as the sympathetic chain,
d) extending along the aorta and spreading out mediodorsally at the level of the kidneys where it often covers the converging iliacs and the renal veins (Smith and Roberts, 1964).

Thus the brown adipose tissue is strategically located around major blood vessels and nerve tracts in the thoracic and cervical regions. It is also close to the spinal cord all the

way down to the kidneys. The hyperplasia of the brown adipose tissue observed during the process of cold-acclimation is known to occur to all the areas described (Smith and Horwitz, 1969).

In spite of the fact that the contribution of this tissue to total heat production is relatively small, this contribution is, however, important because of the distribution of the heat produced. In fact, Smith and Horwitz (1969) have postulated that brown adipose tissue can act as a thermal blanket protecting the vital organs against cooling due to the distribution of the heat produced.

Moreover it has been suggested that the brown adipose tissue, in particular the interscapular pad, is a thermoregulatory organ. This function results from its special vasculature and anatomical location. Smith and Roberts (1964) have shown that Sulzer's vein provides a short, direct channel for the transfer of heat from the venous drainage of the interscapular brown adipose tissue to the spinal cord. Therefore it has been postulated that this anatomical arrangement has an important physiological role which is the suppression of shivering heat leaving the interscapular brown adipose tissue and chanelled through Sulzer's vein could warm up certain spinal cord thermal receptors which in turn are responsible for the control of shivering (Smith and Horwitz, 1969; Brück and Wünnenberg, 1966; Horwitz, 1972).

In addition to the heat-producing function of brown adipose tissue Himms-Hagen (1969) proposed that this tissue has another

function in the development and maintenance of the adaptation to cold. The evidence for this function is that the surgical removal of the interscapular brown adipose tissue from cold-acclimated rats which subsequently live at room temperature causes a progressive and rapid loss of the enhanced capacity of the acclimated rat to respond to noradrenaline and adrenaline; as mentioned above there is no immediate loss of response (see Figs. 16 and 17).

A similar reduction of 40% of the response to noradrenaline was observed by Leduc and Rivest (1969). These authors also found that there is a loss of cold resistance of these animals. Thus the rats lose their adaptive state more rapidly than the normal rate of deacclimation to cold when interscapular brown adipose tissue is removed (see Fig. 9). That this reduction is due not to a change in the sensitivity, but rather to a decrease in the maximum capacity to respond to noradrenaline is shown in Figure 18 (Himms-Hagen et al, 1972).

These experiments indicate that interscapular brown adipose tissue in some way influences the capacity of other tissues to respond to catecholamines. It has therefore been postulated that interscapular brown adipose tissue secretes a factor which acts upon these other tissues.

More direct evidence for this endocrine function of the brown adipose tissue was obtained when it was demonstrated by Himms-Hagen (1970) that the implantation of the interscapular brown adipose tissue in the peritoneal cavity considerably

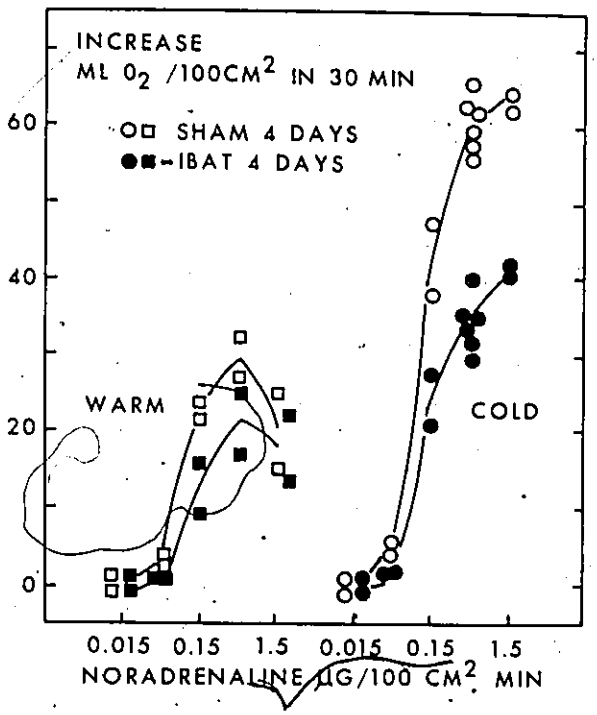


Figure 18. Dose-response curves for the calorigenic effect of noradrenaline in rats from which the interscapular brown adipose tissue was removed 4 days previously (-IBAT) (solid black symbols) and in sham-operated rats (open symbols). Both warm-(squares) and cold-acclimated (circles) rats are shown. The cold-acclimated rats remained at room temperature during the 4 days following the operation.

(From: Himms-Hagen et al, 1972)

reduces the loss of the adaptive state induced by removal of interscapular brown adipose tissue (see Fig. 19).

Removal of interscapular brown adipose tissue prior to cold-exposure of warm-acclimated rats has not prevented the acclimation to cold. However, although such rats eventually develop a normal enhanced calorigenic response to noradrenaline the initial development is delayed about one week, as can be seen in Figure 20 (Himms-Hagen et al, 1972). This apparent lack of effect can be explained in terms of the remarkable adaptive ability of the rats to switch a function from one site to another. Himms-Hagen et al (1972) have shown clearly this point; during the weeks in the cold there is normally, in intact rats, a considerable increase in the size and protein content of the brown adipose tissue, about a third of this increase occurring in the interscapular pad. However, in rats lacking the interscapular pad the cold-induced increase in protein found in other brown adipose tissue deposits is greater than in intact rats and at least partially compensates for the missing interscapular brown adipose tissue, as can be seen in Figure 21.

The endocrine role of brown adipose tissue has been questioned by Horwitz and Smith (1972) who measured the magnitude of the thermogenic response in cold-acclimated rats having had Sulzer's vein ligated and in cold acclimated rats having had interscapular brown adipose tissue removed. The lack of difference in the calorigenic response to noradrenaline in both group and the lack of necrosis in the interscapular pad of the ligated

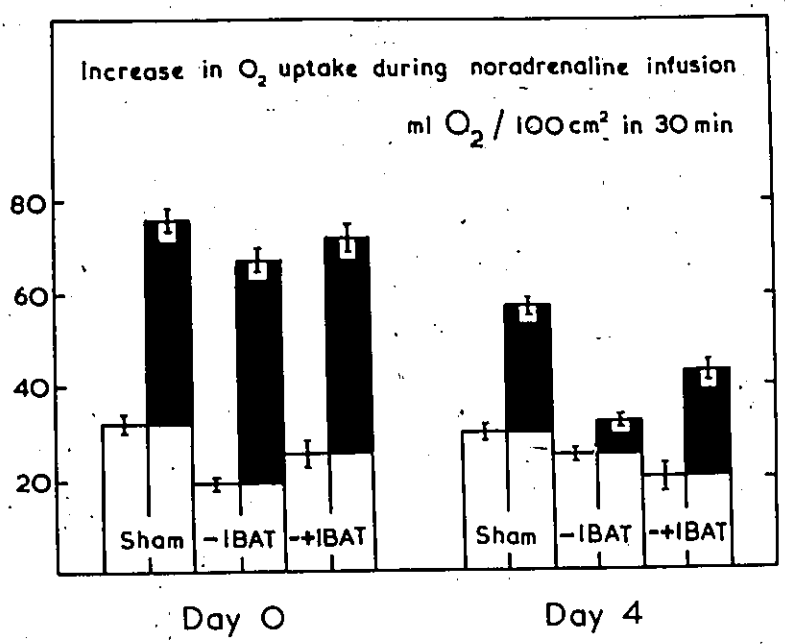


Figure 19.

Effect of removal and replacement of the interscapular brown adipose tissue of warm-acclimated and cold-acclimated rats on the calorigenic response to noradrenaline. Values shown represent the total increase in oxygen uptake during a 30 min infusion of noradrenaline. Each bar is the mean of 4 experiments ± standard error of the mean. Cold-acclimated rats are represented by the bars with the black upper portions and warm-acclimated controls are represented by the open bar to the left. The blackened portion of the bar is the difference between the response of the cold-acclimated rats and the response of the corresponding control warm-acclimated rats; it is a measure of the acclimation to cold. Cold-acclimated rats had lived in the cold for 10-13 weeks; warm-acclimated rats had lived at room temperature during this same period. Rats were removed from cold or warm rooms on day 0, anaesthetized with ether and operated upon as follows: (i) sham-operated, (ii) interscapular brown adipose tissue removed, (iii) interscapular brown adipose tissue removed, cleaned and weighed, cut into 4 or 5 portions and replaced in the peritoneal cavity; a similar laparotomy was performed also on rats in groups (i) and (ii). Rats remained at room temperature until the infusion of noradrenaline.

(From: Himms-Hagen, 1970)

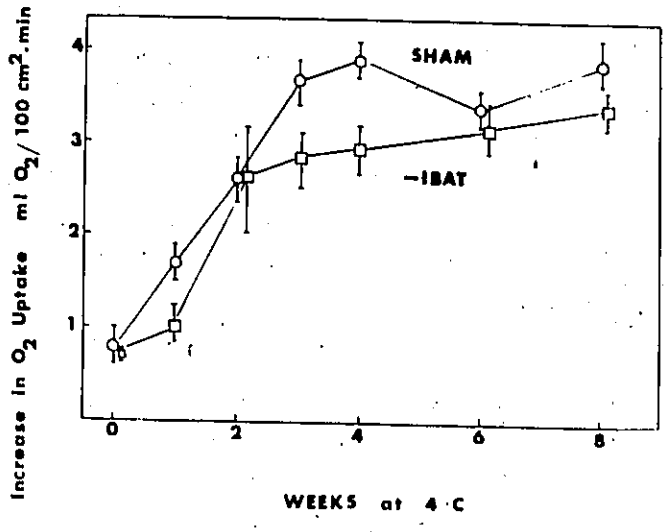


Figure 20. Effect of prior removal of interscapular brown adipose tissue on the development of the enhanced calorigenic response to noradrenaline during acclimation to cold. One week before zero time (the day on which rats were placed in the cold at 4°C) rats either had their interscapular brown adipose tissue removed under ether anaesthesia (-IBAT) or were sham-operated (SHAM). Weights of all rats at this time were: sham,226.3 ±20.7 g; -IBAT, 243.8 ± 26.8 g. Rats remained at 4°C for up to 8 weeks at which time they weighed:306.5 ± 24.9 g; -IBAT, 334.3 ± 19.1 g. At 1,2,3,4,6,and 8 weeks some rats were removed from the cold;after 24 hours at room temperature they were lightly anaesthetized with sodium pentobarbital and their oxygen consumption before and during infusion with noradrenaline was measured. After the infusion rats were killed and all their brown adipose tissue removed, weighed,and protein content measured (see Fig.21).

(From: Himms-Hagen et al, 1972)

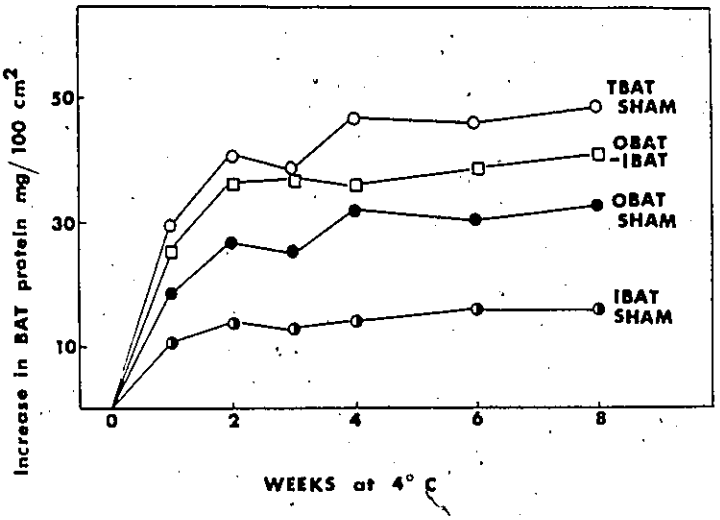


Figure 21. Increase in protein content of brown adipose tissue during acclimation to cold of sham-operated rats and of rats from which interscapular brown adipose tissue was removed prior to the acclimation period. The increase in protein content above that present at time zero is plotted against the duration of cold-acclimation. Shown for the sham-operated rats are the increases in interscapular brown adipose tissue and in other deposits (OBAT), as well as the sum of these increases which represents the rise in the protein content of all the brown adipose tissue in the body (TBAT). Rats from which the interscapular brown adipose tissue was removed 1 week before zero time (-IBAT) (see legend to Fig.20) possessed only the OBAT.

(From: Himms-Hagen et al, 1972)

animals led these authors to consider inconsistent the proposed endocrine function of the brown adipose tissue. That is, any agent secreted by the brown adipose tissue should still be available to the body via drainage through the bilateral thoracodorsal vessels in the animals which Sulzer's vein ligated. Therefore they repostulate that the brown adipose tissue may exert a regulatory influence on the response of the animal via local warming of thermosensitive areas in the spinal cord (Smith and Horwitz, 1969). However the experiment of Himms-Hagen et al (1972) described in Figure 20, in which the removal of interscapular brown adipose tissue prior to the cold-exposure does not prevent the acclimation to cold shows also that the special location of the interscapular pad is not essential for the acclimation to cold to occur. Moreover when the same experiment was performed in this laboratory (Himms-Hagen, unpublished results) the ligated interscapular brown adipose tissue presented a very marked edema. Obviously blocking the principal point of blood draining from interscapular brown adipose tissue could provocate a blood stasis inside the organ so that the secretion of the factor could also be impaired.

Flattery and Sellers (1972) also found that the removal of the interscapular brown adipose tissue prior to cold-exposure, does not impair the development of the cold-acclimation, but as they failed to see the compensatory hyperplasia of the other brown adipose tissue deposits, they suggest that brown adipose tissue does not secrete a factor. However they performed only a visual test in order to check the hyperplasia in the remainder

of the brown adipose tissue. They also removed the interscapular brown adipose tissue from cold-acclimated rats and replaced the animals in the cold after the operation (whereas Himms-Hagen, 1969, placed similar treated rats at room temperature in order to study cold-deacclimation). They observed that during the four next weeks there was no significant change in urinary excretion of the catecholamines or in the calorigenic response to noradrenaline between the operated and non-operated groups of rats. However, since their animals continued to live in the cold, it is quite likely that the remaining brown adipose tissue deposits assumed the role of the interscapular brown adipose tissue which had been removed.

The increased rate of oxygen consumption by cold-acclimated, adult mice after subcutaneous injection of noradrenaline has been measured by Hayward and Davies (1972) for intact animals and for those with their interscapular brown adipose tissue ligated. An immediate reduction of 40% of the calorigenic response to noradrenaline was noted in the mice with their interscapular pad ligated, Since this reduction cannot be ascribed to the loss of an amount of brown adipose tissue constituting less than 0.5% of the body weight, this result supports, to a certain extent, the hypothesis that brown adipose tissue can influence the calorigenic response of other tissues. However, it should be noted that there is one discrepancy between this experiment and the experiment of Himms-Hagen (1969); Hayward and Davies (1972) observed an immediate reduction in the calorigenic response to noradrenaline after removal of the inter-

scapular brown adipose tissue; this discrepancy may be due to the different species used.

As a summary, Himms-Hagen (1972) presented a hypothesis of the role of brown adipose tissue during acclimation to cold. Her ideas are illustrated in Figure 22, which shows in diagrammatic form the growth of brown adipose tissue during adaptation to cold and the progressive increase in "secretion" of heat (having as a consequence the progressive inhibition of shivering, by means of the local warming of centres in the spinal cord) and in secretion of the postulated factor (having as a consequence a progressive increase in the capacity of other tissue to respond to noradrenaline; i.e., the progressive development of nonshivering thermogenesis): all these changes are shown as occurring as a consequence of the excessive and prolonged stimulation of this tissue by noradrenaline.

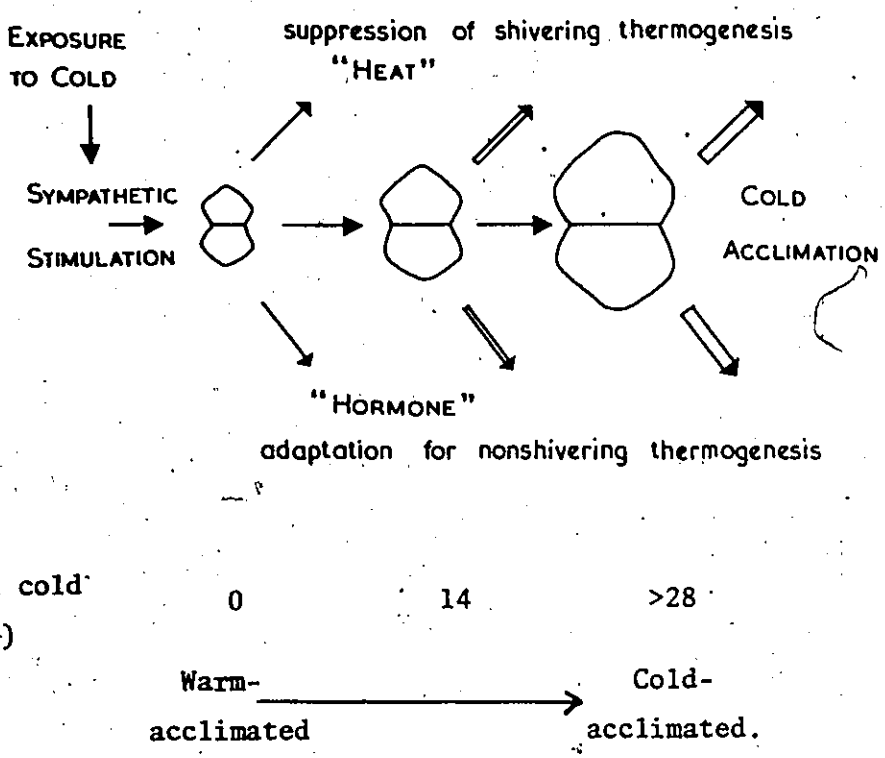


Figure 22. Diagrammatic representation of the proposed progressive increase in heat-producing and hormone-producing capacities of the interscapular brown adipose tissue (IBAT) of the rat during acclimation to cold. After 4 weeks in the cold shivering is completely suppressed and the adaptation for nonshivering thermogenesis is at its maximum.

(From: Himms-Hagen, 1972)

Section F: NONSHIVERING THERMOGENESIS: Current concepts and rationale for the experimental approach described in this thesis.

Nonshivering thermogenesis is an exaggerated calorigenic response to noradrenaline which occurs principally in brown adipose tissue and skeletal muscle of cold-acclimated rats. Despite intensive investigation the nature and mechanism of nonshivering thermogenesis are still not known.

In order to discuss the different possibilities, it is necessary at this point to discuss the factors which control the operation of the mitochondrial respiratory chain in vivo. This is because the main site of oxidative metabolism and of oxygen consumption is the mitochondrial respiratory chain. Of the energy contained in a biological substrate, part is stored in the form of ATP and the rest is released as heat by complete oxidation of the substrate to carbon dioxide and water. Thus the oxygen consumption of a cell is a true reflection of its heat-producing capacity. The availability of ADP in the cell is the primary factor influencing the rate of electron transport, and therefore oxygen uptake. An increase in oxygen uptake could be explained in two basic ways: a) an increased availability of ADP. Since ADP is derived from ATP an increase in the supply of ADP can occur only because of an increase utilization of ATP, e.g., in reactions which require energy. This is the normal mode of respiratory control in coupled mitochondria; b) an increased degree of dissociation of cellular

respiration from oxidative phosphorylation. The respiratory control becomes independent of the concentration of ADP as in the case of uncoupled or loosely coupled mitochondria.

Prusiner and Poe (1968) have proposed a biochemical classification of thermogenic processes:

a) Degradation of ATP. The respiration is controlled at the level of ATP degradation. Since ADP is derived from ATP an increase in the supply of ADP can only occur if there is an increased utilization of ATP for energy-consuming reactions. The mitochondrial respiration is highly coupled to the phosphorylation of ADP and is controlled by its rate of regeneration. Shivering thermogenesis is a good example of this type of thermogenic process. The rapid muscle contractions utilize ATP and thus provide ADP which permits the acceleration of mitochondrial respiration.

b) Oligomycin-insensitive respiration (but sensitive to uncouplers). Oligomycin inhibits coupled respiration by blocking oxidative phosphorylation. The residual respiration is called oligomycin-insensitive and is not under the control of the ADP level. An uncoupler like dinitrophenol can release the oligomycin effects on respiration but not on ATP synthesis. Thus, in coupled mitochondria there exist respiratory pathways which are not coupled with phosphorylation of ADP. It has been suggested these represent increased utilization of high-energy intermediates of the electron transport system for energy-consuming reactions other than phosphorylation, such as ion translocation. Lehninger (1970) showed that the active

transport of divalent cations like calcium is insensitive to oligomycin but blocked by uncoupling agents.

c) Loose-coupling. Loosely coupled mitochondria have normal P:O ratios but a decreased respiratory control. Moreover, their respiration is relatively unaffected by oligomycin or dinitrophenol which decrease their P:O ratios. Their respiration can be controlled by substrate supply.

d) Uncoupling. Uncoupled mitochondria no longer have the capacity for oxidative phosphorylation. The respiration of uncoupled mitochondria is not affected by ADP, oligomycin or dinitrophenol. It seems to be mainly controlled by substrate supply.

In their original paper, Prusiner and Poe (1968) place in the last category the mechanism of nonshivering thermogenesis. This seems very unlikely because the tissues involved in nonshivering thermogenesis require the normal supply of energy in the form of ATP for their normal functions. In the brown adipose tissue, moreover, the need for ATP is greater during the development to the acclimation to cold because the tissue increases its mass and protein content during that period, as was indicated above.

Because of the lack of shivering and because no other metabolic pathway which would result in increased utilization of ATP has been found in the adaptive state of cold-acclimation the first mechanism can also be discarded as a mechanism involved in nonshivering thermogenesis.

Of the other forms, b) and c) probably represent two aspects of the same process, because an increased utilization of some of the high-energy intermediates for a purpose other than phosphorylation of ADP would result in an apparent loosening of coupling. There is evidence for the existence of this type of mechanism in brown adipose tissue, which has been extensively studied. The evidence which supports this mechanism has been reviewed by Himms-Hagen (1970 and 1972) and will only be summarized here. However, skeletal muscle, the major site of nonshivering thermogenesis, appears to have been neglected and little is known about the mechanism of nonshivering thermogenesis in this tissue.

i) Brown adipose tissue. The mechanism of loose-coupling seems to be responsible for the nonshivering thermogenesis process in brown adipose tissue. In fact, some work (Andersen et al, 1970) indicates that the loosening of coupling of brown adipose tissue mitochondria which follows cold-exposure of guinea pigs is a process that gradually develops during cold-acclimation and reverts slowly to normal when the animals are returned to ambient temperature.

Noradrenaline stimulates the adenyl cyclase which is present in the cell membrane (Muirhead and Himms-Hagen, 1971; Skála et al, 1970 and 1972). One of the early events associated with this metabolic activation is a depolarization of the cell membrane. This has been observed both in vivo (Horwitz et al, 1969) and in vitro (Girardier et al, 1968). The cyclic AMP which is produced as a result of the stimulation of adenyl

cyclase accelerates lipolysis, presumably by activating a triglyceride lipase. Brown adipose tissue stores a large amount of triglyceride. The increased supply of free fatty acids provided by the accelerated lipolysis is not only used as a fuel for the maintenance of the high metabolic rate but also plays an important role in regulating heat production by the mitochondria. The free fatty acids appear to cause a controlled loosening of oxidative phosphorylation resulting in an increase in the rate of respiration (Hittelman et al, 1969; Williamson, 1970; Lindberg et al, 1970; Prusiner, 1970).

It is known that free fatty acids can uncouple oxidative phosphorylation and inhibit respiration in several tissues, including brown adipose tissue; brown adipose tissue is more sensitive than other tissues to low concentrations of fatty acids (Rafael et al, 1969). More recently, Bulychev et al (1972) found that sucrose-isolated brown adipose tissue mitochondria were uncoupled because they contained a large amount of free fatty acids. When a minor fraction of these fatty acids was removed the mitochondria were transformed to a coupled state. This and other observations led the authors to suggest that a very small and specific endogenous fatty acid fraction is involved in the regulation of the coupling-uncoupling mechanism of brown adipose tissue. Apparently the mode of action of the fatty acids involves a conformational change of mitochondrial membrane proteins.

It has also been suggested that changes in the relative concentrations of adenine nucleotides within the mitochondria

of brown adipose tissue may be part of a mechanism regulating the coupling of respiration to phosphorylation (Pedersen and Gray, 1970; Skaane et al, 1972); it was suggested that the nucleotides could act on the energy-conserving mechanism of the mitochondria via some conformational change in the mitochondrial membrane. More recently Christiansen et al (1973) have presented a hypothesis in which these two views are combined. These authors suggest that free fatty acids are not only responsible for the loose-coupled state of the mitochondria but also, via the acyl CoA formed from them in the cytoplasm, inhibit the adenine nucleotide translocase of the inner mitochondrial membrane. The consequent alteration in the mitochondrial adenine nucleotide concentration also contributes to the regulation of the coupling of respiration to phosphorylation.

An additional mechanism for nonshivering thermogenesis in brown adipose tissue has been proposed by Horwitz (1973), namely, an acceleration of the Na^+-K^+ ATPase of the plasma membrane. This suggestion is based upon the finding of 60% inhibition by ouabain, an inhibitor of the Na^+-K^+ ATPase, of the noradrenaline-induced calorigenic response in isolated cells (Horwitz, 1973) and of a direct stimulation by noradrenaline of the Na^+-K^+ ATPase in the brown adipose tissue homogenates (Herd et al, 1970). However, Fain et al (1973) failed to find the same effect of ouabain. These authors suggest that the well-known antilipolytic effect of ouabain could also account for the inhibition of the calorigenic effect of noradrenaline.

Thus the exact role of an accelerated Na^+-K^+ ATPase in nonshivering thermogenesis remains in doubt while a definite role for free fatty acids as regulators of mitochondrial respiratory function appears to be well established.

ii) Skeletal muscle. Skeletal muscle has been not as extensively studied as brown adipose tissue, therefore nothing can be said in biochemical terms about the mechanism of nonshivering thermogenesis in this tissue. Only some unspecific changes have been noted during cold-acclimation, in a variety of tissues, including skeletal muscle. Klain (1963) described increments in cytochrome c in heart and skeletal muscle but also in liver, kidney, lung and spleen in cold-acclimated rats. Also it has been reported that cold-acclimated rats show an increase in the specific activities of gastrocnemius and liver cytochrome oxidases and succinic dehydrogenase, but muscle exhibits the greater increase (Hannon, 1960).

Adenyl cyclase in skeletal muscle has not been characterized very well although it was shown to be present in this tissue some years ago (Sutherland et al, 1962; Seraydarian and Mommaerts, 1965). More recently it has been reported by Severson et al (1972) that adenyl cyclase in skeletal muscle resides in the plasma membrane.

There is no difference in the amount or in the catecholamine-sensitivity between the adenyl cyclase of skeletal muscle of warm-acclimated rats and that of cold-acclimated rats. However, during the first few days of exposure to cold there is a trans-

ient increase in the specific activity of the noradrenaline-stimulated, adrenaline-stimulated and the fluoride-stimulated adenyl cyclase of skeletal muscle and an increased sensitivity of adenyl cyclase to stimulation by adrenaline (Letarte-Muirhead, 1972). These transient increases were suggested to occur in association with shivering rather than with nonshivering thermogenesis.

In contrast to brown adipose tissue cells the individual skeletal muscle cells appear to lack direct adrenergic innervation; however, the blood vessels of skeletal muscle contain adrenergic nerves (Fuxe and Sedwall, 1965). Noradrenaline released from these vasoconstrictor nerve fibers may, by diffusion, reach the skeletal muscle cells in concentrations which at least locally may be high. Therefore adenyl cyclase of skeletal muscle may well be activated by noradrenaline, but as was shown above, the enhanced capacity for a calorigenic response to noradrenaline and the increased capacity for nonshivering thermogenesis, which both occur in cold-acclimated rats, are not associated with any change in the amount, properties or operation of the adenyl cyclase system.

iii) Role of mitochondrial protein synthesis in nonshivering thermogenesis. Since muscle appears to be a major site of nonshivering thermogenesis in the cold-acclimated rat, it can be assumed that there is an alteration in muscle which permits the enhancement of the calorigenic response to noradrenaline by this tissue. This alteration does not reside in the initial

stage of interaction of noradrenaline with the muscle, i.e., in the adenyl cyclase system. Since the mitochondria are using the oxygen, it is logical to assume that an alteration in mitochondrial function might provide a basis for non-shivering thermogenesis. Two lines of evidence derived from work in this laboratory suggest that altered synthesis and degradation of mitochondrial proteins occurs in association with cold-acclimation. The first line of evidence is the inhibition of the development of the adaptation for nonshivering thermogenesis during cold-acclimation by inhibition of mitochondrial protein synthesis with oxytetracycline (OTC) (Himms-Hagen, 1971). The second line of evidence is the decreased half-life of certain mitochondrial proteins in tissues in which nonshivering thermogenesis occurs, namely, skeletal muscle and brown adipose tissue (Bukowiecki and Himms-Hagen, 1971; Bukowiecki, 1972).

Mitochondrial protein synthesis is sensitive to inhibition by certain antibiotics such as chloramphenicol and oxytetracycline. It is well known that mitochondria resemble bacteria in the type of DNA, RNA and ribosomes they contain and in the response to antibiotics that specifically inhibit 70-S ribosomal protein synthesis, such as chloramphenicol and oxytetracycline (for review, see Rabinowitz and Swift, 1970; Ashwell and Work, 1970). Although the mitochondrion cannot be considered a fully autonomous organelle, at least some of the mitochondrial proteins seem to be synthesized on the mitochondrial ribosomes,

specifically those associated with insoluble fractions (Beattie, 1971). More recently it has been determined that proteins synthesized by mitochondrial ribosomes are integrated into specific parts of the inner membrane (Werner and Neupert, 1972).

Selective inhibition by antibiotics of brown adipose tissue mitochondrial protein synthesis in rats undergoing cold-acclimation was thought to be possible because of studies showing that chloramphenicol appears to affect principally rapidly dividing tissue (Firkin and Linnane, 1969; Gonzalez-Cadavid et al, 1970). Also several recent reports show that chloramphenicol can inhibit the synthesis of cytochrome oxidase and of the inner mitochondrial membrane of regenerating rat liver without inhibiting the regeneration of the liver cells (Firkin and Linnane, 1969; Kroon and de Vries, 1970; De Vries and Kroon, 1970; Gonzalez-Cadavid et al, 1970). As the in vivo studies of De Vries and Kroon (1970) indicated that oxytetracycline also had the same specific inhibitory action as chloramphenicol but was metabolized more slowly than chloramphenicol and produced its effects during a long period of time oxytetracycline was chosen in the studies carried out by Himms-Hagen (1971).

When rats living at 4°C were treated with oxytetracycline for 2 weeks, the normal increase in the specific activity of the cytochrome oxidase of the brown adipose tissue was abolished, as is shown in Figure 23 (Himms-Hagen, 1971). In this case the specific activity of cytochrome oxidase was used as an index of the amount of inner membrane present in brown adipose tissue mitochondria. Figure 23 also shows that no effect of the anti-

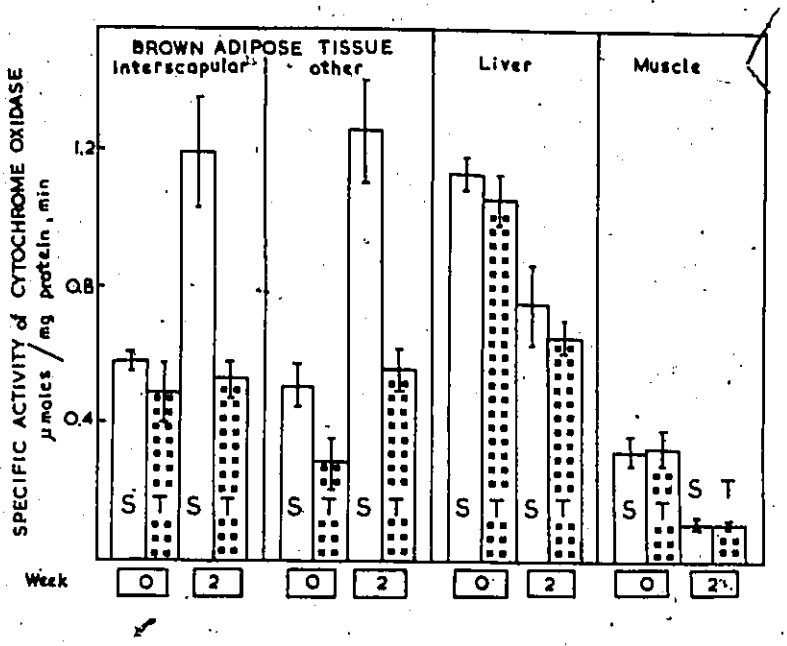


Figure 23. Effect of oxytetracycline on the specific activity of cytochrome oxidase (used as an index of the amount of mitochondrial protein) in brown adipose tissue, liver, and muscle of rats before (week 0) and 2 weeks after (week 2) acclimation to cold. Oxytetracycline (Terramycin, Pfizer, 250 mg in 3 ml) was administered im, twice daily, for 2 weeks; the total dose was 200 mg/kg per day (T). Control rats received im injections of saline (S).

(From: Himms-Hagen, 1971)

biotic on liver or skeletal muscle cytochrome oxidase activity was observed. In spite of the marked effect of oxytetracycline upon brown adipose tissue cytochrome oxidase, neither the growth of the rats nor of the brown adipose tissue (as indicated by increase in wet weight and protein content) was altered by the antibiotic treatment (see Fig. 24). In the same oxytetracycline-treated rats, the development of the enhanced calorigenic response to noradrenaline was also markedly inhibited as is shown in Figure 25. Thus, increased synthesis of a small fraction of brown adipose tissue mitochondrial proteins appears to be necessary for the development of acclimation to cold. The use of an inhibitor of mitochondrial protein synthesis has given a new approach to the study of nonshivering thermogenesis.

A second and more direct approach to the study of the role of mitochondrial protein metabolism in cold-acclimation has been the measurement of protein half-life in mitochondria of warm-acclimated and cold-acclimated rats (Bukowiecki and Himms-Hagen, 1971). The experiment was performed by labelling mitochondrial proteins with leucine-^{14}C and measuring the rate of disappearance of the label from the proteins. A significant decrease was found in the half-lives of some mitochondrial proteins from brown adipose tissue and skeletal muscle of cold-acclimated rats in comparison with warm-acclimated rats, as can be seen in Figure 26. The decrease in the half-lives of mitochondrial proteins occurs in the relatively insoluble "contractile" and "structural" proteins fractions (the names used for the different mitochondrial fractions do not imply their function:

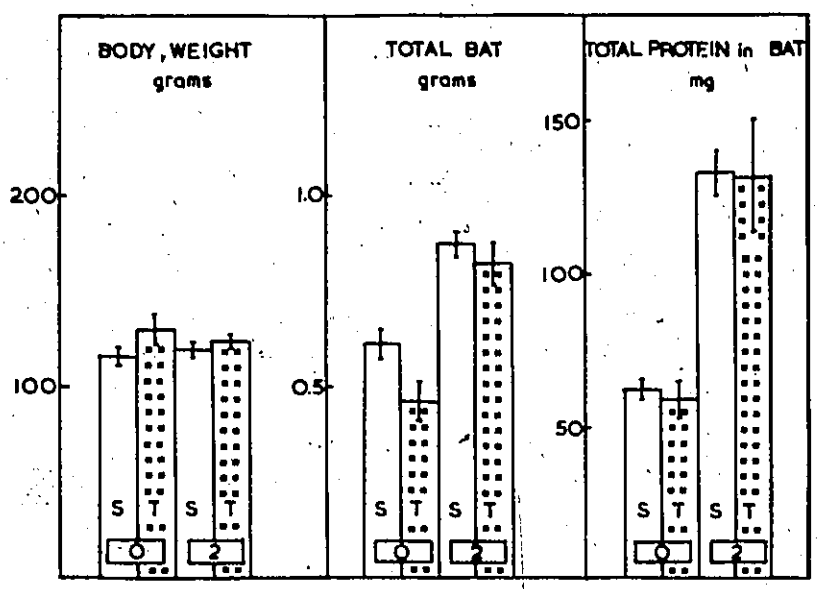


Figure 24. Effect of oxytetracycline on the body weights, wet weight of total brown adipose tissue (Total BAT), and total protein content of brown adipose tissue of rats during acclimation to cold. S = saline-treated rats ; T = oxytetracycline- treated rats. For other details see legend to Fig.23.

(From: Himms-Hagen, 1971)

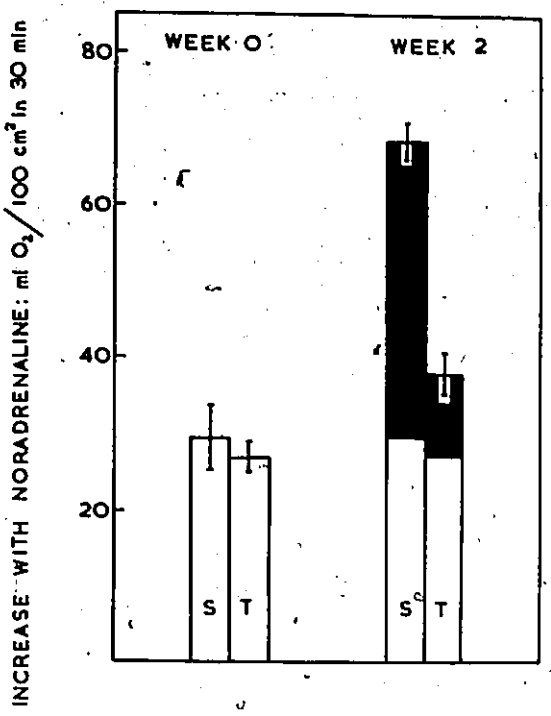


Figure 25. Effect of oxytetracycline administration during acclimation to cold on the calorigenic response to noradrenaline. S= saline-treated rats, T= oxytetracycline-treated rats (See legend to Fig 23). The black portion of the bars represents the enhancement of the calorigenic response to noradrenaline due to the 2 weeks of cold-acclimation.

(From: Himms-Hagen, 1971)

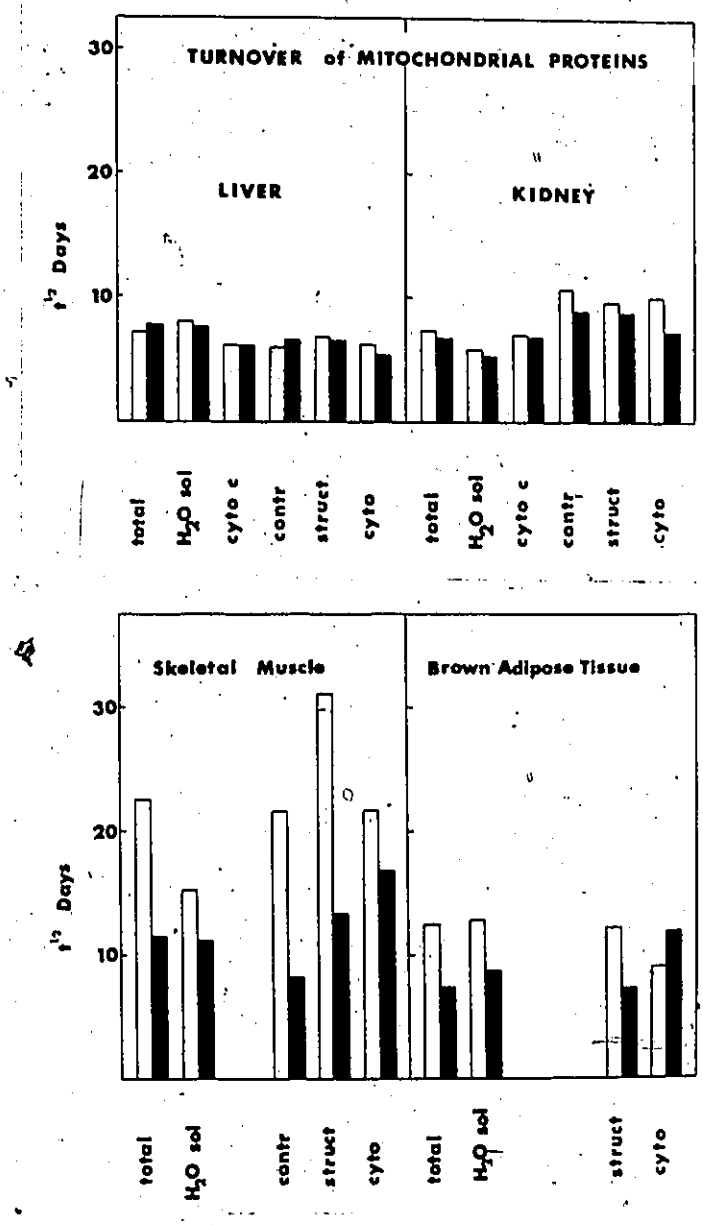


Figure 26. Half-lives of different groups of mitochondrial proteins of liver, kidney, interscapular brown adipose tissue, and skeletal muscle of warm-acclimated and cold-acclimated rats. The half-life (in days) is given for total mitochondrial proteins and for five different groups of mitochondrial proteins in tissues of warm-acclimated (open bars) and cold-acclimated (black bars) rats.

(From: Bukowiecki and Himms-Hagen, 1971)

they come from the technique used in their isolation, according to their solubility properties, in agreement with the protocol of fractionation described by Beattie et al, 1966, 1967 a and 1967 b). Figure 26 also shows that no change in the half-lives of the various kidney or liver protein fractions occurs.

Depocas (1966) had previously shown that cytochrome c from skeletal muscle does not turn over more rapidly in cold-acclimated rats. This fraction accounts for only 5% of the total protein and is synthesized on the cytoribosomes (Gonzalez-Cadavid and Campbell, 1967; Kadenbach, 1970). The lack of effect of cold-acclimation on the half-lives of mitochondrial proteins in liver is in agreement with the findings of Lusena and Depocas (1966 and 1967).

The specific decrease in the half-lives of certain mitochondrial proteins in tissues where nonshivering thermogenesis is known to take place, such as skeletal muscle and brown adipose tissue, and lack of change in other tissues which are not important sites of nonshivering thermogenesis such as liver and kidney, suggest that the decrease might be associated with the development of the increased capacity for nonshivering thermogenesis and that mitochondria of these tissues may be different in cold-acclimated rats compared to warm-acclimated rats. This decrease in the half-lives of brown adipose tissue and skeletal muscle mitochondria is associated mainly with the insoluble mitochondrial protein fractions which, as was explained before, are believed to be synthesized at least partially by the mitochondrial protein system. The decrease may be due to a change

in the rate of synthesis and/or degradation of one or more proteins in the group of proteins or to a change in the amount of one or more proteins in the group, or it may be associated with both changes.

It should be pointed out that this observation of a decreased half-life of certain mitochondrial proteins in muscle of cold-acclimated rats provides the first indication of a biochemical difference between skeletal muscle of cold-acclimated rats and skeletal muscle of warm-acclimated rats.

iv) Rationale for the experimental approach described in this thesis. This thesis describes a study of the changes in muscle mitochondria induced by acclimation to cold and associated with nonshivering thermogenesis. This study is based on the following considerations:

1. Muscle is the major site of nonshivering thermogenesis.
2. The biochemical basis of nonshivering thermogenesis is unknown; no changes in muscle structure or function have been found except the change from shivering thermogenesis to nonshivering thermogenesis.
3. It is assumed that there must be a change in muscle associated with the development of the capacity for nonshivering thermogenesis. This change is not detectable by measuring known biochemical reactions; in this the change resembles shivering thermogenesis, which also is not detectable by

measuring biochemical reactions.

4. The changes to be sought are based upon recent findings of certain properties of the adaptation for nonshivering thermogenesis.
   a) inhibition of mitochondrial protein synthesis with oxytetracycline inhibits the development of the adaptation (Himms-Hagen, 1971)
   b) there is a decrease in the half-life of some mitochondrial proteins of skeletal muscle and brown adipose tissue but not of liver and kidney (Bukowiecki and Himms-Hagen, 1972)
   c) brown adipose tissue has a role in the maintenance of the adaptation, presumably in skeletal muscle (Himms-Hagen, 1969).
5. Since cold-acclimation involves the above mentioned changes in muscle mitochondrial protein synthesis it seems likely that this would be reflected in a change in the appearance of the mitochondria. Nonshivering thermogenesis obviously occurs in the mitochondria and may well be based upon a change in the structure and/or function of the mitochondrion itself.

Therefore the aim of this research has been to find a difference in the muscle mitochondria of cold-acclimated rats and to correlate such a difference with the adaptation for nonshivering thermogenesis by studying it under conditions where the adaptation is:

a) known to occur

b) known to be prevented from developing

c) known to be reversed after its development

d) known to be decaying normally

e) known to be decaying more rapidly than normal

The experimental approach used in this research in order to perform the above study involves:

A comparison of both red and white muscles of cold-acclimated rats with both red and white muscle of warm-acclimated rats in the following ways:

- Electron microscopy of red and white muscles
- Fiber characteristics and distribution in red and white muscles (assessed by histological estimation of succinic dehydrogenase activity)
- Measurement of number, size and various enzyme activities of mitochondria isolated from muscle.

These procedures were carried out on rats that were undergoing acclimation to cold, fully acclimated to cold, or undergoing deacclimation to cold. In addition, conditions were studied in which the normal acclimation process was modified: by prevention of the acclimation to cold with oxytetracycline, by reversal of the acclimation to cold with oxytetracycline, by acceleration of the deacclimation to cold by removal of the interscapular brown adipose tissue. The last type of experiment was hoped also to give some support to the suggested endocrine role of the brown adipose tissue during the development and maintenance of acclimation to cold.

Because of the nature of the experimental approach it is necessary at this point to review the normal characteristics and distribution of skeletal muscle fiber types. Also will be summarized the normal ultrastructure of muscle with special reference to the mitochondria.

Section G. THE FIBER TYPES OF MAMMALIAN SKELETAL MUSCLE

It has long been known that the skeletal muscles of an individual mammal differ in color and that the fibers of which skeletal muscle is composed differ in their microscopic appearance (for review see Gauthier, 1970; Close, 1970).

Histochemical procedures for localizing enzymic activity have confirmed these early observations and have extended them by revealing additional differences among muscle fibers. Table I shows some of the enzymic activities used in the characterization of the fiber types in mammalian skeletal muscle.

In addition, electron microscopic ultrastructural criteria are now available for the identification of fiber types which correspond to those distinguished by enzyme histochemistry (Gauthier and Padykula, 1966; Shafiq, Gorycki and Milhorat, 1969; Gauthier 1969 and 1970).

Even within a given muscle, the fibers appear to differ from one another in several characteristics. This subject has been reviewed recently (Gauthier, 1970; Close, 1970). Here will be summarized only some of the principal characteristics of mammalian skeletal muscle, with special reference to the rat.

As a result of morphological, histochemical and electron microscopical observations three principal types of fiber that differ in ultrastructure, in metabolic profile and in other biochemical parameters, have been described for mammalian skeletal muscle. Several systems of nomenclature have been proposed; some of them are listed in Table I, together with the principal morphological and histochemical properties. In spite of the

large number of classifications proposed by several authors there is general agreement that there are three basic morphologically, physiologically and biochemically distinct types of muscle fibers in the skeletal muscle of mammals.

In the discussion that follows, and in the remainder of this thesis the nomenclature proposed by Gauthier and Padykula (1966) of "red", "white" and "intermediate" muscle fibers is used.

The histochemical properties listed in Table I indicate that white fibers have high glycolytic, low oxidative and high myofibrillar ATPase activities, that the intermediate fibers have low glycolytic, intermediate oxidative, and low myofibrillar ATPase activities, and that the red fibers have intermediate glycolytic, high oxidative and high myofibrillar ATPase activities.

Mitochondrial content is a particularly suitable cytological criterion for the identification of fiber types. When procedures known to demonstrate mitochondrial enzymes (mitochondrial ATPase, succinic dehydrogenase) or the phospholipid components of mitochondria (with Sudan black) are used, a characteristic cytological pattern is observed which corresponds closely with the sites occupied by mitochondria in equivalent sections examined with the electron microscope (Gauthier, 1970).

As can be seen in Table I, mitochondrial content is inversely related to the diameter of the fibers. Fibers with a small diameter are rich in mitochondria, especially along the periphery of the fibers. These are the red fibers. On the other hand the white fibers have the largest diameter and have the lowest

TABLE 1. Morphological and histochemical types of fiber in mammalian skeletal muscle.

CLASSIFICATIONS:			
Gauthier and Padykula (1966)	White	Intermediate	Red
Olson and Swett (1966)	A	C	B
Ashmore and Doerr (1971)	α-white	β-red	α-red
Barnard et al (1971)	Fast-twitch white	Slow-twitch intermediate	Fast-twitch red
Dubowitz and Pearse (1960)	II	I	II
Peter et al (1972)	Fast-twitch glycolytic	Slow-twitch oxidative	Fast-twitch oxidative-glycolytic
Burke et al (1971)	FF	S	FR
Romanul (1964)	I	III	II
Stein and Padykula (1962)	A	B	C
Brooke and Kaiser (1970)	II B	I	II A
Yellin and Guth (1970)	α	β	αβ

...continued

TABLE I (continuation)

MORPHOLOGICAL PROPERTIES:			
Mitochondrial content (1,2)*	Small	Intermediate	Large
Z line. (1,2,3)	Narrow	Intermediate	Broad
Fiber diameter (1,3,4)	Large	Intermediate	Small
Neuromuscular junction (3)	Large and complex	Intermediate	Small and simple
Sarcoplasmic reticulum (1,5)	Compact arrangement of broad parallel tubules or flattened sacs		Elaborate open network of narrow tubules extends across the myofibrils
HISTOCHEMICAL PROPERTIES:			
Distribution of succinic dehydrogenase (1,3,4,5)	Even network	Even network	Predominantly subsarcolemmal
Oxidative enzyme activities (6,7,4,8,9,10)	Low	High or intermediate	Intermediate or high
Glycolytic enzyme activities (7,8,6,9,10)	High	Low	Intermediate or low
Mitochondrial ATPase (1)	Low	Intermediate	High
Myofibrillar ATPase at pH 9.4 (11,6)	High	Low	High

...continued

TABLE 1 (continuation)

Mitochondrial α-glycero-phosphate dehydrogenase (8)	High	Low	High
Myoglobin content (8,7)	Low	High	High
Glycogen content (8,12)	Intermediate	Low	High
Cytochrome a (8)	Low	Intermediate	High
Cytochrome c (8)	Low	Intermediate	High

* References for Table I.
1) Gauthier (1969); 2) Shafiq et al (1969); 3) Padykula and Gauthier (1970); 4) Stein and Gauthier (1962); 5) Gauthier (1970); 6) Eversole and Standish (1970); 7) Romanul, (1964); 8) Peter et al (1972); 9) Nolte and Pette (1972); 10) Ariano et al (1973); 11) Edgerton and Simpson (1969); 12) Gillespie et al (1970).

mitochondrial content. The intermediate fibers, as their name indicates, have characteristics intermediate in these respects.

Despite the known striking manifestations of heterogeneity however, differences between fibers within a particular muscle have not always been taken into consideration in studies of altered muscle function. Normal differences between fiber types have indeed been incorrectly interpreted as an alteration in muscle structure due to an experimental procedure used.

In studying the adaptive changes that take place in skeletal muscle, especially in the mitochondria, during the development and maintenance of cold-acclimation it is essential to avoid the possibility of misinterpretation mentioned above. It is, therefore, necessary to consider in detail the normal ultrastructural features of the different types of muscle fiber in order to appreciate any changes which may take place during acclimation to cold.

Usually, if the proportions of the red fibers predominates the muscle is called "red muscle" and so on. However, this classification into "red" and "white" muscle is arbitrary and imprecise because it does not recognize the complex heterogeneity of muscle fiber distribution and the varying proportions of them in the total muscle. Some muscles have homogeneous distribution of fibers but others do not. A heterogeneous distribution could produce a mistake in the classification or in the interpretation of experimental data. These problems can be solved by working with muscles which are composed almost or entirely of only one type of fiber. As an example, some types

of muscle can be indicated which approximate this condition. Of the skeletal muscles of the guinea pig, the red vastus has 78% of red fibers, 18% of white fibers and 4% of intermediate fibers, its gross appearance is red (Peter et al, 1972). The white vastus has 29% of red fibers and 71% of white fibers, its gross appearance is white. The soleus has 100% of intermediate fibers and its gross appearance is red.

In the rat it is much more difficult to find a muscle even with such homogeneity (Ariano et al, 1973). However, one muscle of the rat is divided clearly into red and white portions. This muscle is the semitendinosus. The normal fiber distribution and ultrastructure of this muscle has been extensively studied by Gauthier (1969 and 1970). Using the histochemical demonstration of succinic dehydrogenase activity as a measure of mitochondrial content, it can be demonstrated that the red portion of the semitendinosus consists primarily of fibers rich in mitochondria (red and intermediate fibers) while the white portion consists almost entirely of fibers with a low mitochondrial content (white fibers) (Gauthier, 1969, see Table II).

Table II shows that the red fibers of the semitendinosus are the smallest and the white fibers the largest. Red and intermediate fibers are numerous in the red band, and together they constitute 92% of the population. In the white band, however, there is a total of only 18% of these two types. Gauthier (1969 and 1970) also studied by electron microscopy the ultrastructural appearance of the three fibers. In the red fiber, large mitochondria with closely packed cristae are abundant

TABLE II. Analysis of fiber population in the rat semitendinosus

Region of muscle	Type of fiber	Fiber diameter (average)	% of fiber type	Area contributed by each fiber type (% of total area)
RED BAND	Red	45μ	52	44
	Intermediate	52μ	40	44
	White	59μ	9	13
WHITE BAND	Red	47μ	4	2
	Intermediate	47μ	14	7
	White	69μ	82	91

(from Gauthier, 1970)

beneath the sarcolemma and in longitudinal interfibrillar rows. Additional paired mitochondria are present at the I bands. The intermediate fiber is similar except that subsarcolemmal and interfibrillar mitochondria tend to be smaller and fewer in number. Z lines are clearly thinner in the intermediate than in the red fiber. In the white fiber, mitochondria are sparse; usually there are no subsarcolemmal aggregations or interfibrillar rows, and the paired mitochondria at the I bands are the predominant type. Z lines are narrowest in the white fiber. All these characteristics and others are also listed in Table I.

The semitendinosus muscle of the rat is thus one of the muscles very well characterized for this species in the distribution, proportions and ultrastructure of its three fiber types. By taking advantage of the clear separation of this muscle into red and white portions it is possible to analyze separately the ultrastructure of red and white muscle fibers within a single muscle. This makes the semitendinosus a very useful muscle with which to carry out studies during cold-acclimation. This muscle has, therefore, been used in all the histochemical and ultrastructural studies described in this thesis.

In summary, three distinct types of skeletal muscle fibers can be identified consistently on the basis of morphological, physiological and biochemical parameters. Therefore, in order to evaluate physiological and ultrastructural studies it is necessary to recognize the heterogeneity of the muscle fibers and to characterize the muscle in the distribution and proportions of these three different types of fiber. This should be

kept in mind for the discussion in the next section of the adaptive changes which have been described as taking place in muscle mitochondria under different experimental conditions.

Section H. ADAPTIVE CHANGES IN MUSCLE MITOCHONDRIA IN RELATION TO CHANGES IN MUSCLE FUNCTION

When a rat is placed in a cold environment a large increase in heat production occurs and contributes to the maintenance of body temperature. During the initial two to three weeks in the cold the increase in metabolic rate is largely due to shivering in the muscles. As the adaptation for nonshivering thermogenesis appears, reaching a maximum after about 4 weeks in the cold, so shivering decreases, disappearing entirely at this time. The metabolic rate of the muscles of the cold-acclimated rat is as high as that of the muscles of the non-acclimated rat but the way in which the increase in metabolic rate is produced is entirely different in these two states. In the shivering muscles the initial stimulus comes from the motor nerve to the muscle and causes the muscle contraction of shivering. In the muscle of the cold-acclimated rat the increase in metabolic rate is initiated by noradrenaline, that is, non-shivering thermogenesis is an enhanced calorigenic response to noradrenaline.

Other stimuli which increase muscle activity, for example, electrical stimulation or periodic exercise (training) are known to cause structural and biochemical changes in the muscle, particularly in the muscle mitochondria. It is logical, therefore, to seek a change in muscle mitochondria occurring as a consequence of continual shivering. However, the elevated metabolic rate of muscle in the cold-acclimated rat in the cold

could equally well be associated with biochemical and structural changes in the mitochondria. Other conditions known to raise the metabolic rate of muscle, such as hyperthyroidism, are associated with biochemical and structural changes in the muscle mitochondria. It is not known whether the transient period of shivering at the start of acclimation to cold could influence the mitochondria of the muscle in some way that enables them to be prepared for the development of the nonshivering thermogenic process.

There follows a short review of all those conditions known to produce adaptive changes or alterations in muscle mitochondria. Among them, can be cited: exercise, stress which produces overload, electrical stimulation, denervation (cross-innervation) and aging. As many studies have been performed with heart rather than with skeletal muscle, this organ is also included here. Also are included the few studies carried out in skeletal muscle in cold-acclimated and cold-exposed mammals.

a) EXERCISE

It is attempted here to evaluate the adaptive changes that occur in muscle mitochondria as consequence of physical activity. However, there is a great confusion in the literature regarding the effects of exercise upon muscle mitochondria. Some of the confusion results from incorrectly considering all forms of exercise as qualitatively identical stimuli to which skeletal muscle fibers adapt uniformly. It is necessary to distinguish between:

1) Short-term exercise which can be light or rigorous depending on the type (swimming, running), time of duration of the stimulus (minutes or hours), and other characteristics.
2) Long-term exercise or training which also can be light, mild or rigorous depending on the type (swimming with or without overload, running, weight lifting, etc.), time of duration of the stimulus (weeks or months), frequency (once, twice a day, etc.) and other characteristics such as age, nutritional state, etc.

Another cause of confusion derives from the fact that the effect of some types of exercise has been incorrectly evaluated in muscles which do not participate directly in the activity imposed by the exercise.

Also, as was indicated in the preceeding section, the different cytological characteristics of the muscle fibers have not been considered by some authors and normal differences between red and white fibers have been erroneously attributed to adaptive changes.

In view of this confusion in the literature only those studies which clearly specify the type of exercise, duration, work involved, name and type of muscle used have been selected for discussion in this thesis.

Short-term exercise

Light short-term exercise does not usually produce any change in muscle mitochondria. In order to see some effect it

is necessary to increase the intensity and duration of the exercise, usually to exhaustion.

It has been shown that acute and exhaustive exercise, such as swimming until exhaustion, provokes a marked increase in the mitochondrial mass of cardiac muscle of the dog (Laguens et al, 1966) and also of the rat (Laguens and Gomez-Dumm, 1967). Swimming also provoked marked changes in the fine structure of the mitochondria of the heart muscle; after 90 and 120 minutes of exercise, the mitochondrial mass of the cardiac cells increased significantly. The results of Laguens and co-workers have been confirmed by Pelosi and Agliati (1968) and King and Gollnick (1970). The increase in mitochondrial mass is apparently due to hypertrophy of the individual mitochondria rather than to an increase in the number of the mitochondria. Laguens and co-workers postulate that during the mitochondrial hypertrophy there is an active replication of mitochondrial components. They also showed that under the stimulus of acute and exhaustive exercise the mitochondria of the myocardial cells are able to synthesize DNA (Laguens, 1968). Therefore in order to inhibit this first step of mitochondrial replication they used acriflavin (Laguens et al, 1972). It was then shown that there was no increase in the mitochondrial mass after acriflavin treatment plus exercise, suggesting that mitochondrial DNA synthesis is involved in the increase of mitochondrial mass.

Thus short-term exercise produces adaptive changes in myocardial mitochondria. There is an active replication of some mitochondrial components, perhaps necessary for the maintenance of the high metabolic demand in this condition.

In skeletal muscle, in contrast to heart muscle, no significant changes in the number and in the size of mitochondria occur in gastrocnemius muscle of rats which were swimming until exhaustion (Gollnick and King, 1969 a and 1969 b). However by using a more strenuous exercise such as running it was possible to see a swelling of the mitochondria and some degree of degeneration (Gollnick and King, 1969 b; Gollnick et al, 1971). Thus skeletal muscle under the stress of short-term exercise cannot develop adaptive changes in the mitochondria.

Long-term exercise or training

It has been known for a long time that a vigorous program of prolonged exercise, such as long distance running or swimming induces a gross hypertrophy of muscle and/or an increase in the diameter of the muscle fibers together with an increase in the capacity of the animal to perform exercise (for review see Gordon, 1967). Actually the programs of training are designed in such a way that there is no gross hypertrophy, but adaptive changes can be observed in the muscle of the animals submitted to this program.

Table III summarizes the effects of long-term exercise upon the muscle fiber characteristics. It can be seen that exercise maintained for a long time usually induces an increase in the percentage of red fibers and a corresponding decrease in the proportion of white fibers. The soleus muscle, an intermediate type, apparently was not affected in any case, perhaps due to the fact that is not involved in the physical activity.

TABLE III. Effects of long-term exercise upon fiber characteristics.

Animal Type of training Work and duration Reference	Muscle	Fiber size and fiber proportions
Rat Swimming (overload) 30 min/day; 52 days Edgerton et al. (1969)	Soleus Plantaris	No changes Increase in red fibers
Rat Running voluntarily 5 hr/day; 6 weeks Weight lifting 9.0×10^8 ergs/day; 6 weeks Kowalski et al. (1969)	Quadriceps	Increase in red fibers
Guinea pig Running, different times and speed; 18 weeks Barnard et al. (1970)	Gastrocnemius	Increase in red fibers Decrease in white fibers
Guinea pig Running 30-60 min/day; 8 weeks Faulkner et al. (1971)	Plantaris	Increase in the number of total fibers, decrease in the diameter and more red fibers
Guinea pig Running 30-60 min/day; 8 weeks Lieberman et al. (1972)	Diaphragm	No change in fiber size, increase in red fibers.
Guinea pig Running 30-60 min/day; 8 weeks Maxwell et al. (1973)	Soleus and Psoas ... Plantaris	No change in fiber distribution, increase in the size ... Increase in red fibers
Rat Running 2 hr/day; 12 weeks Baldwin et al. (1972)	Soleus ... Quadriceps Gastrocnemius	No change ... Increase in red fibers Decrease in white fibers

Discrepancies are evident in the effect of exercise on the size of the fiber; reduction, no change or an increase are reported in different cases depending on the type of exercise, intensity imposed and muscle studied.

In general, there is an increase in the proportions of fibers having high oxidative enzyme activities. This appears to be one mechanism through which muscle can adapt to a chronic repetitive exercise. The increase in red fibers indicates an increase in the number and/or the size of mitochondria.

That the adaptive changes observed in muscle fibers are reversible if the training is stopped has been documented by Faulkner et al (1972) who showed that after several weeks of detraining the changes observed in the muscle fibers regress toward control values.

Table IV shows the effects of training upon the ultrastructure of mitochondria and upon various biochemical parameters of mitochondrial function in skeletal muscle.

Electron microscopic studies reveal that after a long period of training the mitochondria from skeletal muscle have increased in number and in size and have more densely packed cristae. Also several reports indicate that this is accompanied by an increased oxidative capacity of the mitochondria, due to the increased concentration of respiratory carriers and increased levels of several enzymes of oxidative metabolism. The electron microscopic observations are also supported by the fact that there is an increase in the total protein content of the mitochondrial fraction.

Table IV also shows that swimming is not by itself a strenuous enough exercise for the production of the effects. As will be seen, only cardiac mitochondria, in certain conditions, respond to this mild training.

Exercise to the point of exhaustion has been reported to result in damage to skeletal muscle mitochondria of untrained animals (Gollnick and King, 1969 b; Gollnick et al, 1971). However if the animal has been previously trained the mitochondria are able to support and not be affected by exercise to exhaustion. The increased oxidative capacity of muscle mitochondria, the total yield, P/O ratios and respiratory control indices were not significantly different from control (trained) values after trained rats were submitted to a exhaustive exercise (Terjung et al, 1972). The mitochondria also appeared well preserved on electron micrographs of gastrocnemius muscle from the exhausted animals. This suggests that the adaptive changes that take place in muscle mitochondria after training are of such degree that the increase in the endurance of the animal is due to an increase in the endurance of the organelle itself. If the trained animal becomes exhausted and cannot maintain the exercise, this is due to other causes and not to impairment of function of the skeletal muscle mitochondria.

Another apparently important change (not listed in Table IV) that occurs in muscle after a long-term exercise is the increase in the activity of glutamate-pyruvate transaminase without any change in lactic dehydrogenase activity (Molé et al, 1973). Both enzymes are known to utilize pyruvate, the first

TABLE IV. Effects of long-term exercise on skeletal muscle. Mitochondrial ultrastructure and biochemical parameters.

Animal. Muscle Type of training Work and duration Reference	Mitochondrial Characteristics (Electron Microscope)	Biochemical changes
Rat. Gastrocnemius Swimming 30 min/day; 8 weeks Hearn and Wainio. 1956		No change in Succinic dehydrogenase.
Rat. Mixed muscles Swimming 30 min/day; 6 weeks Gould and Rawlinson.1959		No changes in levels of lactic dehydrogenase, Malic dehydrogenase and phosphorylase.
Rat. Gastrocnemius. Running. Several speeds and times; 3 months Holloszy, 1967.		Increased capacity of isolated mitochondria to oxidize pyruvate. Increase in Succinic dehydrogenase, DPNH-dehydrogenase, DPNH-cytochrome c reductase, Succinic oxidase. Increased concentration of cytochrome c and total protein content.
Rat. Gastrocnemius Running. 1 hr/day; 10 weeks Gollnick and King 1969a and 1969b	More larger mitochondria with more densely packed cristae.	

...continued

TABLE IV. (continuation)

Animal. Muscle Type of training Work and duration Reference	Mitochondrial Characteristics (Electron Microscope)	Biochemical changes
Rat. Tibialis anterior. Swimming 2-3 hr/day; 2 weeks Kraus et al. 1969	Marked increase in mitochondrial size and number, densely packed cristae	Increased capacity of isolated mitochondria to oxidize pyruvate. Increase in glycerol phosphate dehydrogenase, Succinic dehydrogenase. Increase in cytochromes content and mitochondrial protein. Phosphorylation coupled. No change in glycolytic enzymes.
Rat. Gastrocnemius. Running, several speeds and times; 3 months Holloszy et al. 1970		Increase of enzyme of Citric acid cycle.
Rat. Gastrocnemius. Running 1 hr/day; 10 weeks Gollnick et al. 1971.	Increase in number and size with densely packed cristae	Increase in succinic dehydrogenase and mitochondrial protein.
Rat. Gastrocnemius Quadriceps 2 hr/day; 12 weeks Molé et al. 1971		Increase in levels of palmityl CoA Synthase, Carnitine palmityltransferase and palmityl CoA Dehydrogenase, and in the capacity to oxidize fatty acid. Increase in mitochondrial protein.

...continued

TABLE IV (continuation)

Animal. Muscle Type of training Work and duration Reference	Mitochondrial Characteristics (Electron Microscope)	Biochemical changes
Rat. Gastrocnemius Quadriceps Soleus 2 hr/day; 12 weeks Baldwin et al.1972		Increased capacity to oxidize pyruvate and palmitate. Increase in levels of cytochrome oxidase, carnitine palmityltransferase and citrate synthase. Increase in cytochrome c.
Guinea pig. Gastrocnemius Running. Different speeds and times; 18 weeks. Barnard et al. 1970		Increased capacity to oxidize pyruvate, succinate and ascorbate. Coupled phosphorylation. Increased mitochondrial protein.

producing alanine and the second lactate. This finding indicates that during endurance training there is an increased capacity of the mitochondrial glutamate-pyruvate transaminase to compete with the cytoplasmic lactic dehydrogenase for pyruvate. This adaptation, in the view of the authors, could result in the conversion of a greater percentage of the pyruvate formed in the trained muscle during exercise to alanine and less to lactate, which is known to accumulate in untrained muscle during strenous exercise and interfere with muscle function. Therefore this adaptation could improve the endurance of the trained animal to exercise and give a solid explanation to the fact that in trained animals there is less lactate accumulation during exercise.

Table V summarizes the effects of long-term exercise on the ultrastructure of heart mitochondria and the biochemical changes in heart. All the studies report an increase in the number of the cardiac mitochondria. The mitochondria appear with more densely packed cristae and have increased oxidative capacity. All these adaptive changes appear in the heart mitochondria if the training is strong enough (running or swimming for prolonged periods each day). Swimming for a short time each day (Hearn and Wainio, 1956) is not a strong enough stimulus for the production of the adaptive changes.

Discrepancies in reports of the change in size of the mitochondria may be due to different morphometric methods used by different authors. Arcos et al (1968) reported an increase in the size of cardiac mitochondria, but Edington and Cosmas (1972)

TABLE V. Effects of long term exercise on heart. Mitochondrial ultrastructure and biochemical parameters

Animal Type of training Work and duration Reference	Mitochondrial Characteristics (Electron Microscope)	Biochemical changes
Rat Swimming 30 min/day; 8 weeks Hearn and Wainio. 1956		No changes in succinic dehydrogenase activity.
Rat Swimming 6 hr/day; 140-180 hr Arcos et al. 1968	Increase in the size and in the number. More densely packed cristae.	Increase in mitochondrial mass. Increase in oxidative activity.
Rat Running 1 hr/day; 16 weeks Edington and Cosmas. 1972	Increased number of smaller mitochondria.	No increase in mitochondrial mass.
Rat Swimming 6 hr/day; 400-1500 hr. Aldinger and Sohal. 1970	Increase in the number of mitochondria.	
Mice Running 1 hr/day; 4 weeks Hamilton and Ferguson. 1972		Increase in succinic dehydrogenase.

reported an alteration of the mitochondrial population in the direction of more smaller mitochondria.

b) AGING

Little work has been done on the changes in muscle mitochondria that occur during the aging of the animal. This aspect must be carefully considered because generally the programs of training take a long time and obviously the trained animal at the moment of analysis must be compared with a sedentary control of the same age. In the studies reported previously this has been taken in consideration. It is only for purpose of clarity that the changes and alteration of muscle and muscle mitochondria with age is discussed separately.

It has been reported that the senile decrease in weight of skeletal muscle of rats is due to a decrease in number and in the volume of the muscle fibers. The more affected type of fiber are the red ones which show a marked decrease in number (Tauchi et al, 1971). Similar results have been reported for the diaphragm (Lieberman et al, 1972), and the plantaris muscle of guinea pig (Faulkner et al, 1971). This latter report indicates that training prevents the decrease in the number of the muscle fibers and the decrease in the number of the red fibers.

Chen et al (1972) reported that the ADP-stimulated (State 3) respiration of myocardial mitochondria with glutamate-malate, glutamate-pyruvate, palmitylcarnitine and β-hydroxybutyrate as substrates declined in rats after the age of 20

months. There was no significant decline in pyruvate-malate, α-ketoglutarate, palmityl-CoA, succinate and ascorbate cytochrome c oxidation. Skeletal muscle mitochondria from senescent animals showed a similar decline in glutamate-malate oxidation but not in palmityl-CoA, palmitylcarnitine, succinate and ascorbate-cytochrome c oxidation. The controlled oxidation with ADP-limiting (state 4) and the ADP/O ratio were not affected. These results led to the authors to suggest that the control of oxidative metabolism and hence the ATP supply may be altered in aging animals.

Recently Tomanek and Karlsson (1973) studied the myocardial ultrastructure of young and senescent rats. Among several alterations in the myofibrils it was found that foci of numerous mitochondria were frequent in senescent heart, but many of the organelles were unusually small, of irregular shape and randomly orientated. Such foci were usually subsarcolemmal. The authors suggest that the presence of foci of dense mitochondrial accumulations may represent an adaptation, i.e., proliferation of mitochondria, to impaired utilization of oxygen.

c) HORMONAL EFFECTS

Among the hormones known to provoke changes in muscle mitochondria are thyroid hormone and some steroid hormones.

Thyroid hormone

It is known that thyroid hormones control the metabolic rate of homeothermic vertebrates. The respiratory activity of skeletal muscle mitochondria would account for the larger part of the basal metabolic rate (BMR), which is well known to be

under the control of thyroid hormones. Thus control of muscle mitochondrial function by thyroid hormones is very likely. Indeed an increase in the respiratory activity of mitochondria of rat muscle occurs after prolonged treatment with low doses of thyroid hormones (Tata et al, 1963).

It has been reported that the total amount of mitochondria from both thyroidectomized animals and normal animals made hypermetabolic with thyroxine is increased 2.5 to 3.5 times over that of their corresponding controls (Gustafsson et al, 1965). In all cases, there was an increase in the mitochondrial population and the profile ratio of cristae to matrix was also considerably increased, thus indicating both relative and absolute enlargements of the entire surface of the cristae per unit fiber. However, this result has been criticized by Gauthier (1969,1970) who suggests that the authors did not recognize the heterogeneity of the gastrocnemius muscle and that the red fibers were selected in this case as the experimental, leaving the white ones as the control. The gastrocnemius muscle of the rat is known to have equal proportions of red and white fibers (Ariano et al, 1973) but the distribution of them is random and not zonalized as the semitendinosus muscle, therefore it is highly probable that the authors misinterpreted the electron microscope observations. In my opinion the criticism is valid. Moreover in the view of the fact that the structural changes persisted for at least 3 weeks after the cessation of thyroxine treatment by which time the elevated mitochondrial respiratory and phosphorylative activity had declined to normal values and

in view of the similar changes seen in thyroidectomized rats it seems unlikely that the changes reported were related directly to the hyperthyroid state.

Several reports indicate that thyroid hormone does cause some changes in the enzyme activity pattern of energy-supplying metabolism of skeletal muscle and heart of the rat. Of several enzymes examined, hexokinase and mitochondrial glycerophosphate dehydrogenase reveal the most significant increases (Kubista et al, 1971). Histochemical staining for glycerophosphate dehydrogenase revealed that the increased activity of this enzyme is predominantly demonstrable in the subsarcolemmal mitochondria. It appears that the muscle mitochondria acquire an enhanced oxidative capacity due to the treatment with thyroid hormone. Comparable results have also been reported by Lee and Lardy (1965).

In the rat heart Gross (1971) found that thyroidectomy slowed the turnover of mitochondrial DNA and protein and that this slowing could be prevented by treatment of the animal with thyroxine. Gross also reported a significant increase in the total cardiac content of mitochondrial protein in response to the thyroxine treatment. This work has been extended by McCallister and Page (1973), their results showing not only that additional mitochondria are accumulated in response to thyroxine, but also that these mitochondria are characterized by a denser packing of the cristae. Similar results were obtained by Reith et al (1973).

Steroid hormones

Several reports indicate that an excess of glucocorticoids produces various alterations in muscle in general and in the mitochondria themselves. Smith (1964), using histochemical methods, reported predominant involvement of white muscle fibers in myopathies induced in rabbit muscle by triamcinolone administration. In contrast, in a combined light and electron microscopic study, D'Agostino and Chiga (1966) reported that red fibers in the rabbit diaphragm were most affected by cortisone administration. In order to clarify the problem, the effects of triancinolone and cortisone on rat soleus, a red muscle, and on the medial head of the gastrocnemius, a white muscle, were investigated with the electron microscope by Tice and Engel (1967). In both muscles mitochondrial alterations predominated. Mitochondria appeared with increasing frequency and become more regularly oriented with respect to the fibrils. Also these mitochondria presented more densely packed cristae. After the early phase of mitochondrial proliferation many fibers, especially in white muscle, contained circumscribed areas from which most or all of the interfibrillar mitochondria had been lost. Several degenerative foci in the fiber structure were also observed.

As the studies reported above generally involved the daily administration of glucocorticoids for long period of time, Bullock et al (1971) made a study of the morphology of the (predominently white) muscle vastus lateralis of the rat after a single high dose of triamcinolone. It was observed after this treatment that there was a pronounced enlargement and pro-

liferation of the mitochondria in the zone adjacent to the subsarcolemmal zone. There was, in addition, some destruction of mitochondria in deeper zones and some evidence of fragile membranes in the enlarged mitochondria.

The cause of the proliferation of enlarged mitochondria through steroid action is unknown. Attempts to correlate the mitochondrial changes with an alteration in muscle ribosomes isolated from the steroid treated rats have failed (Bullock et al, 1972).

d) ELECTRICAL STIMULATION

It is known that if the skeletal muscle is electrically stimulated for short or long periods of time several physiological and biochemical changes take place.

Salmons and Vrbova (1969) have found that the speed of contraction of a fast muscle (white) can be changed by continous stimulation of the motor nerve over a period of weeks; this produces a marked slowing of the time course of contraction and relaxation (to that of red muscle).

The electrical stimulation of fast muscles not only produce a change in the physiological characteristics of the muscle but also a biochemical change, namely the synthesis by white muscle of myosin characteristic of red muscle (Streter et al, 1973).

It is known that myosin isolated from red skeletal muscle of the rabbit differs in several aspects (ATPase activity and different subunits) from myosin isolated from white skeletal muscle and these characteristics correlate closely with its contile speed.

Intermittent long-term stimulation of fast rabbit muscle for up to 28 days with a frequency pattern resembling that of a slow muscle led not only to a slowing of the time course of contraction already during the first week (Pette et al, 1973) but also to a rearrangement of activities of key enzymes of energy supplying metabolism. Initially a decrease of extramitochondrial enzymes of glycogenolysis and glycolysis and energy rich phosphate transfer together with a decrease of mitochondrial glycerolphosphate dehydrogenase were observed. Large initial increases were found in enzymes involved in glucose phosphorylation and fatty acid activation. Later an increase of key enzymes of the citric acid cycle and fatty acid oxidation as well as ketone bodies utilization could be shown. The authors suggest that a change in mitochondrial population has taken place under the influence of the stimulation. They also remark that the changes they observe are different in several respects from those produced by endurance training such as running. In keeping with the changes noted above, histochemical staining of succinate dehydrogenase showed that after the stimulation the mosaic-like fiber composition of the fast muscles was transformed into a more uniform population in which there was a total absence of the white fibers. With regard to fiber size, however, small and large fibers could still be distinguished in the cross-section of the stimulated muscle. The smaller and somewhat darker stained fibers seem to correspond to the red fibers in the control muscle; their total number does not appear to have increased. The size of the larger fibers

which represent the majority, resembled that of the intermediate type in the control muscle.

e) CROSS-INNERVATION

It is known that cross-innervation of red and white muscles results in a qualitative and quantitative alteration of many of the specific biochemical and physiological properties of these muscles.

The effect of cross-innervation has been studied by several groups. Yellin (1967) reinnervated the soleus muscle (red) with nerve fibers that normally supply white muscle. He observed a fairly extensive conversion of the soleus fibers, so that the reinnervated muscle possessed groups of fibers of histochemical types not found normally in the soleus. Romanul and Van Der Meulen (1966, 1967) conducted experiments in which the nerve of a fast muscle (flexor digitorum longus) reinnervated slow muscle (soleus) and vice-versa. The cross-innervated muscles reversed their speed of contraction and the enzymatic characteristics of their fibers. Thus the high oxidative and low glycolytic enzymatic profile of the soleus muscle fibers was changed to the low oxidative and high glycolytic pattern of normal flexor digitorum longus. A considerable amount of information is available on the dynamic properties of the contractile material of cross-innervated muscles, specially those related on the properties of myosin have been reviewed by Close (1972). Some of the biochemical quantitative changes after cross-innervation have been reviewed by Guth (1968). However, no ultrastructural studies on changes in mitochondria are available.

f) OVERLOAD

Overloading a muscle generally results in hypertrophy and therefore changes in the mitochondria under the influence of the overload can be expected.

A number of investigations have indicated that mammalian muscles change their contractile properties following tenotomy (tendon section) but in general this technique produces fiber atrophy and several abnormalities in the muscle organization. On the other hand following tenotomy of muscle(s) in the hind-limb, there is a rapid compensatory hypertrophy of the synergist muscle. This technique therefore can be used in the study of muscle hypertrophy.

Unilateral tenotomy of the gastrocnemius muscle in normal rats leads to rapid compensatory growth of the synergistic plantaris and soleus muscles (Jablecki and Kaufman, 1973). It was found that in the hypertrophied muscles the total myosin was increased but no enzymatic (ATPase) or structural differences were found. To increase the overload on the soleus the authors also performed a double tenotomy (gastrocnemius plus pantaris); in this condition the soleus muscle hypertrophies faster and presents a decrease in myosin ATPase. There are no reports on the oxidative capacity of the hypertrophied muscle. It would be of interest to study the ultrastructure and biochemical parameters of muscle mitochondria in the early stage of hypertrophy.

Considerable information has accumulated about some of the biochemical and cellular processes that accompany cardiac

hypertrophy. A variety of experimental procedures have been developed which produce in animals a rapid or gradual increase in cardiac mass in response to increased work demand. These include constriction of the ascending or descending aorta or main pulmonary artery; renal or deoxycorticosteroid-induced systemic hypertension; damage to valves; chronic hypoxia, anemia and others (for review see Rabinowitz and Zak, 1972; Rabinowitz et al, 1971; Schwartz, 1971).

In general, cardiac hypertrophy finally results in failure of the organ, a condition which is accompanied by a mitochondrial defect. However, prior to severe heart failure, mitochondria appeared to be functioning normally with respect to energy production. Some work on this aspect has been carried out with rabbit heart in which the hypertrophy was created by progressive stenosis of the ascending aorta (Sordahl et al, 1973). Mitochondria exhibited marked increases in respiratory activity during early stages of hypertrophy, followed by a decrease during later stages. As this decrease leaves insufficient activity to meet the energy demands of the hyperfunctioning heart if contributes ultimately to the failure of the organ. Apparently the mitochondria degenerate after the period of hyperfunction.

Characteristic features of animals subjected to prolonged high-altitude stress are polycythemia and hypertrophy of the heart (Shertzer and Cascarano, 1972). A major alteration of heart mitochondria occurs in this condition. The specific activity of succinic-dehydrogenase increases but that of cytochrome

oxidase remains unchanged. Also spectral analysis of the respiratory chain components reveals no change for cytochromes a+a3 despite increases in cytochromes b, c+c1 and other electron carriers. Morphometric studies performed with the electron microscope reveal that the heart mitochondria under this condition increase in size proportionally to the organ enlargement (Herbener et al, 1973).

g) DENERVATION

When the motor nerve supplying a vertebrate skeletal muscle is transected the muscle initially hypertrophies and then rapidly atrophies (Pellegrino and Franzini, 1963). The denervated muscle shows a progressive decrease in weight and the fiber diameter decreases. Ultrastructural observations reveal several abnormalities in the myofibrils and the mitochondria disappear in parallel with the advancing degenerative process.

There are several conflicting reports of the detailed nature of the changes in skeletal muscle after denervation. Some important points to be noted are that the alterations observed may be related to the time interval following denervation, or to the age of the animal and once again to the fact that there has been little recognition of the fact that skeletal muscle is composed of different types of fibers. Therefore here will be presented only the structural and cytochemical features of mammalian skeletal muscle fibers following denervation of the rat semitendinosus after 14 days of operation (Gauthier and Dunn, 1973). The usual cytochemical criteria for identifying

fiber types could not be used after the denervation; there was a shift toward a more homogeneous population of fibers which were rich in mitochondria, but which had an ultrastructural appearance distinct from that of normal fibers, but lacked the characteristic subsarcolemmal aggregations of mitochondria. The authors suggest that the apparent increase in the proportion of red or intermediate fibers is due to a preferential alteration of white fibers which become degraded rather than being due to a transformation of one fiber type to another. The presumed survival of red or intermediate fibers is not understood.

h) COLD-EXPOSURE AND COLD-ACCLIMATION

It has long been known that after cold-acclimation at 6°C white rats have a smaller muscle mass than control animals of the same age kept at 30°C (Héroux and Gridgeman, 1958). Water and protein determination in soleus and gastrocnemius muscles as well as in the whole muscular mass revealed that the reduction in the muscle mass after cold-acclimation is a real tissue difference due to a reduced protein deposition and not only a water content difference (Héroux, 1958). The same report also shows that there is a decrease in the size of the fiber but an increase in the number of them.

Histological changes occurring in the gastrocnemius, soleus and plantaris muscle of rats on exposure to cold stress has been reported, in which a reduction is observed in the diameter of the muscle fibers of rats exposed to daily cold stress for 2 hours for two weeks (Scaria et al, 1965). The reduction in muscle fiber diameter was accompanied by an increase in the

concentration of myoglobin, succinic dehydrogenase and cytochrome oxidase. The authors suggest that this enables the participation of more and more fibers in the oxidative activity of the muscles by transforming white fibers into red ones. However the data presented give no direct proof of this statement. Moreover, these changes are more likely to be due to the effects of long-term exercise because of the technique used for the cold stress. The rats were kept in cold water at 15°C. They were prevented from drowning by suspending them with a loose noose. The authors reported that the rat struggled in the cold water for the first 10-15 min, after which they remained motionless throughout the period of exposure. After they were taken out of the bath they shivered violently for 1-2 hours. Therefore, this experiment cannot be classified among those of simple cold-exposure, neither among those of cold-acclimation due to the fact that the rats were shivering. In any case, the effects described look more like those obtained with long-term exercise.

In spite of the leading role of skeletal muscle in nonshivering thermogenesis there have been few studies of skeletal muscle of cold-acclimated rats. It is, therefore, one of the aims of this thesis to explore muscle from cold-acclimated rats by using histochemical, biochemical and ultrastructural techniques. Since nonshivering thermogenesis is a condition in which there is a high metabolic rate, as in the condition of exercise, obviously some adaptive changes can be expected to occur in the muscle mitochondria.

CHAPTER III: MATERIAL AND METHODS

Section A. MATERIALS

1. Rats

Male white rats purchased from Holtzman Company were used in all the experiments described in this thesis. After arrival in the laboratory they were kept at room temperature (25-28°C) for one week in a large wire cage containing 12 rats. At the beginning of the experiment, they were divided into the groups required for the specific experiment and placed into individual wire cages with free access to water and food. The temperature of the cold room was maintained at 4°C and the temperature of the warm room was 25-28°C. Artificial light was kept on for approximately 12 hours daily (6 a.m. to 6 p.m.). The rest of the time the animals were in the dark. The body weight was determined weekly, with the exception of those treated with daily doses of oxytetracycline which were weighed twice a day in order to adjust the doses of the antibiotic to the body weight. Only rats with normal growth were used in the experiments.

2. Chemicals

Adenosine-5'-triphosphate (disodium salt), Tris (tris-hydroxymethyl-aminomethane), EDTA (ethylene diamino tetra-acetic acid, di or tetrasodium salt), Nitro-blue tetrazolium, noradrenaline bitartrate (L-arterenol), Rotenone (Grade II), sucrose, mannitol, β-DPNH (β-dihydrodiphosphopyridine nucleotide, disodium salt), cytochrome c (type III), heparin (177 units/mg,

protease (subtilisin BPN'; 6.6 units/mg), Hepes buffer and bovine serum albumin were purchased from Sigma Chemical Company. Propylene oxide, glutaraldehyde (50% in H_2O), and uranyl acetate were purchased from J.T. Baker Chemical Company. Araldite resin 502, DDSA (Dodecenyl succinic anhydride), DMP-30 (Dimethyl amino methyl phenol) and formvar solution were purchased from Ladd Research Industries. Osmic acid (OsO_4) was purchased from Engineered Materials. Lead citrate was purchased from Electron Microscopy Sciences. Copper grid (3 mm, 200 mesh) from E.F. Fullam Inc, and empty gelatin capsules (N° 00) were purchased from Lilly Company.

Section B. METHODS

1. Removal of the organs

The rats were killed by decapitation at room temperature after determination of their body weight. The length of time that the different groups of rats spent in the warm room or in the cold room is indicated for each experiment.

Immediately after decapitation the organ or organs were removed. The liver, the interscapular brown adipose tissue (IBAT), the other deposits of brown adipose tissue (OBAT) and skeletal muscle were quickly removed, weighed and transferred into their respective ice-cold mitochondrial isolation medium or frozen in plastic vials.

For the histological and electron microscope preparation of samples the semitendinosus muscle was removed as described by Gauthier (1969); the medial aspect of the upper hindlimb

musculature was exposed, and the gracilis posticus muscle was cut and retracted to gain access to the semitendinosus muscle which was cut at both end. A small part of the muscle was divided longitudinally in the red and white portion and immersed in the pre-fixative for electron microscopy. The rest of the semitendinosus was tied by both end to a flat wooden splint and submerged in liquid nitrogen in order to perform the histological estimation of succinic dehydrogenase.

2. Histological estimation of succinic dehydrogenase

The frozen muscle was placed in a cryostat (-20°C) for sectioning. Using a rotary microtome, sections 10 μ thick were cut and mounted upon untreated clean glass slides.

Succinic dehydrogenase activity was localized using Nitro-blue tetrazolium, according to the method of Nachlas et al (1957). The sections mounted on the glass slides were incubated in a Coplin jar containing 10 ml of the incubation medium which consisted of: phosphate buffer (0.20 M), pH 7.6, 1 part, sodium succinate (0.20 M) 1 part and Nitro-blue tetrazolium (1 mg/ml), 2 parts. The incubation medium was prepared just before use. The incubation was conducted at 37°C for 10 minutes. The sections were rinsed in saline solution, fixed with 10% neutral formalin for 5 min, washed and mounted in glycerol-gelatin ready for the light microscope. Pictures were taken at several magnifications.

3. Electron microscopy

The electron microscopy studies were performed in accord

with the following procedure.

a) Prefixation: The samples of red and white semitendinosus after removal from the rat were submerged in ice-cold 4% glutaraldehyde (prefixative) in 0.1 M phosphate buffer, pH 7.4. The muscle then was cut with a razor blade in long strips following the longitudinal axis of the fiber. The strips were cut transversely to form small rods of 1 mm diameter and 1.5 mm long. The operation was performed on a sheet of solid wax under a drop of cold prefixative. The small rods of muscle were placed in a test tube containing 5 ml of fresh prefixative and left in ice until a total of two hours were completed from the initial submersion of the muscle in the prefixative. The prefixed samples were rinsed three times (20 min each) with 0.1 M phosphate buffer pH 7.4.

b) Fixation: The phosphate buffer was poured off and the tissue was fixed with 2% OsO_4 in 0.1 M phosphate buffer for 1 hour. The fixative was prepared the night before the experiment and stored in the cold. The fixation also was carried out in an ice-cold bath.

c) Dehydration: The fixative was poured off and the fixed muscle was rinsed with 30% ethanol. The ethanol was poured off and the tissue was submitted to dehydration by passage through an ice-cold ethanol series of 30, 50, 70, 90 and 100%, 7 min each. The last stage of dehydration with 100% ethanol was repeated at room temperature.

d) Embedding: The procedure of embedding was essentially that of Luft (1961). The absolute alcohol was poured off and the tissue samples were immersed in two changes of propylene oxide for 10 min each. The second change of propylene oxide was poured off and 2 ml of fresh propylene oxide was added to the tube. An equal quantity of the freshly prepared resin mixture with accelerator (see below) was added and mixed by swirling. After 1 hour another equivalent volume of the resin mixture (4 ml) was added. At least 12 hours of infiltration were allowed; the tubes containing the samples and the resin mixture were maintained during this period in a rotatory shaker at low speed. The resin mixture contains: Araldite resin 502, 20g;
DDSA, 13g;
DMP-30 (accelerator), 0.46 ml.

e) Preparation of blocks: At least 10 blocks containing samples from the same muscle were prepared by filling a gelatin capsule (N^{o} OO) to 2 mm of the top with a fresh resin mixture. The muscle samples were transferred to the top of the capsule and allowed to sediment to the bottom of the capsule. After 2-3 hrs, the resin mixture with the small sample of muscle was then polymerized according to the following schedule (Luft, 1961):

i) Overnight at 35^{o}C

ii) Next day at 45^{o}C

iii) Next night at 60^{o}C

f) Sectioning: Thick sections (2-3μ) and thin sections (Silver-gold) were cut on a Reichert ultramicrotome (Model UM-2) using glass knifes. The thick sections were stained with toluidine blue and examined with the light microscope in order to orientate the block for the thin sectioning. The thin sections were placed on a copper grid coated with a formvar film.

g) Staining: The sections placed on the grid were stained with lead citrate according to Reynolds (1963) for 20 min, rinsed with distilled water and stained with uranyl acetate for 20 min.

h) Observation: The thin sections were examined with a Siemens Elmiskop-IA electron microscope operating at 80 KVolts. Pictures from at least two blocks and different planes were taken on Kodak plates (6.5 by 9 cm) at 7,000; 15,000; and 20,000 times of magnification.

4. Measurement of oxygen uptake

The measurement of oxygen uptake was essentially that described by Himms-Hagen (1969). The rats were lightly anaesthetized with sodium pentobarbital (2.8 mg/100 cm^2 body surface, administered intraperitoneally) and a polyethylene cannula placed in a tail vein. Sodium chloride solution (0.9 g/100 ml, referred to as saline) was infused at a rate of 0.021 ml/min. For a period of 30 min a solution of noradrenaline was infused at a rate of 0.5 μg/100 cm^2 body surface min. (Noradrenaline was dissolved in 0.01 N hydrochloric acid: this stock solution was diluted in saline immediately before infusion). This dose

produces a maximum calorigenic response; larger doses do not cause any larger effect and frequently kill the animal (Himms-Hagen, 19699. The rat was placed in a metabolic chamber in which the oxygen uptake was measured with an open-circuit system (with a flow rate of 477 ml air/100 cm^2 body surface.min) and a Westinghouse oxygen analyzer. Surface area was calculated according to the formula:

Area (in cm^2) = K x body weight 2/3 Where K = 7.5 (Diack, 1930).

5. Removal of interscapular brown adipose tissue

The interscapular brown adipose tissue (IBAT) was removed under ether anaesthesia. In sham-operated animals a similar skin incision was made under ether anaesthesia but the IBAT was not disturbed. In some experiments the rats were kept at room temperature for up to 5 days following the operation. In those experiments in which the calorigenic response to noradrenaline was studied immediately after the operation a slightly different procedure was followed. The rats were injected with pentobarbital (80% of the usual dose), immediately anaesthetized with ether, and then the operation was performed; the administration of ether was stopped during the operation. Rats did not regain consciousness but remained lightly anaesthetized by the pentobarbital. Infusion of noradrenaline was not started until the resting oxygen uptake was steady and in the normal range; this was usually within 1 hour after the operation.

6. Treatment with oxytetracycline

Male rats weighing 80-90 g were kept for 7 days in groups in large cages at room temperature. They were then divided into several groups according to the specific experiment. Rats of the groups treated with the antibiotic were injected intramuscularly in the legs with oxytetracycline twice daily, at 9 a.m. and 9 p.m. (Terramycin, Pfizer, solution for intramuscular injection containing 250 mg in 3 cm^3); the total dose per day was 200 or 300 mg/kg (DeVries and Kroon, 1970), according to the experiment; control rats were injected twice a day with saline solution. The first injection (of oxytetracycline or saline) was made at 9 p.m. at room temperature and a second injection also at room temperature, was given at 9 a.m. the following day. Some of the rats from the different groups received an infusion of noradrenaline this same day (referred to as zero time). The remainder of the rats from the different groups were placed in the cold or remained in the warm room in individual metal cages. After these rats had been in the cold or/and warm for the desired period, receiving injections twice daily, they were transferred to room temperature after a 9 p.m. injection and thereafter they remained at room temperature in order to recover from the effect of noradrenaline secreted from endogenous sources. They were reinjected at 9 a.m. the following morning and infused with noradrenaline on the same day and processed as described above for the noradrenaline infusion and measurement of oxygen uptake,

When the experiment was designed only to obtain samples for histological studies and electron microscopy the rats were taken directly from the cold (or warm) just before the last injection of oxytetracycline)or saline) at 9 a.m. Also in these rats the injection of oxytetracycline was done twice a day in the right leg, leaving the left leg for the removal of the semitendinosus muscle.

In some experiments after 2 weeks of treatment with oxytetracycline (or saline) some rats were submitted to the opposite treatment for one or two weeks more.

7. Preparation of homogenates and isolation of mitochondria

In those experiments in which it was attempted to use oxytetracycline to prevent the development of the adaptation for nonshivering thermogenesis, to reverse the adaptation after its development and to study biochemical properties of brown adipose tissue, muscle and liver (see section B of Chapter IV) the isolation of mitochondria was carried out systematically and simultaneously from liver, brown adipose tissue and muscle. Liver and brown adipose tissue mitochondria were isolated essentially as described by Weinbach (1961) and skeletal muscle mitochondria as described by Chappell and Perry (1954). The mitochondria from liver and brown adipose tissue were isolated in a medium of 0.25 M sucrose in 0.01 M tris buffer pH 7.4, and the muscle mitochondria in Chappell-Perry medium (without ATP). The chilled tissues, suspended in their respective isolation media, were cut with scissors until a fine mince was obtained.

During all the isolation procedure the temperature of the samples was maintained between 0-4°C. The minced skeletal muscle was homogenized first with a loosely fitting all-glass Potter-Elvehjem homogenizer in several short periods of time, for a total time of about 3-4 min. The other tissues were homogenized directly with tightly fitting homogenizers for a total period of 2-3 min. The homogenates were diluted with their respective isolation medium to a volume of several times the initial weight of the tissue, as follows:

IBAT;	0.15 - 0.50 g;	Volume homogenate: 10 ml
Liver;	5.00 - 12.00 g;	" " 50 ml
Muscle;	2.00 - 3.00 g;	" " 25 ml

Aliquots were taken from the homogenates and frozen in order to estimate later protein content and several enzymic activities. The rest of the homogenate was submitted to differential centrifugation in order to isolate mitochondria.

In those experiments in which a maximal extraction of mitochondria was attempted in order to estimate the number of them the mitochondria from skeletal muscle were isolated by a procedure developed in this laboratory (Himms-Hagen, unpublished results). After removal, the muscles (usually 10 g) were cooled, rinsed in cold 0.15 M KCl, minced with the help of a razor blade, and weighed. The minced muscle was digested during 30 min in an ice-cold beaker: the digestion medium was prepared just before use and was as follows: about 80 ml of Mannitol medium was measured and then heparin (250 U/ml), protease (20 mg) and ATP (60.5 mg) were added to it; the pH was adjusted to 7.4 and

the final volume was brought to 100 ml with Mannitol medium. The Mannitol medium was composed of 0.21 M Mannitol, 0.07 M sucrose, 0.01 M EDTA (tetra sodium salt) and 0.01 M Hepes buffer pH 7.4. After the 30 min period the digested muscle was homogenized with a loosely fitting all-glass Potter-Elvehjem homogenizer in lots of 10 ml each for short periods of time. The homogenates were pooled, mixed and an aliquot of 5 ml was used for the immediate estimation of cytochrome oxidase activity. The rest of the homogenate was submitted to differential centrifugation as follows:

a) The homogenate was diluted with mannitol medium (with heparin, 250 U/ml) and centrifuged at 650 g for 5 min.
b) The supernatant was filtered through gauze and centrifuged at 13,000 g for 15 min.
c) The mitochondrial pellet was resuspended in mannitol medium (without heparin) and homogenized smoothly by hand in a glass homogenizer with a teflon pestle.
d) The resuspended mitochondrial fraction was centrifuged at 10,000 g for 10 min.
e) The resulting mitochondrial pellet was resuspended in a small volume of mannitol medium (without heparin) and centrifuged at 650 g for 5 min. The supernatant was kept on ice and the sediment was resuspended in mannitol medium (without heparin) and centrifuged at 650 g for 5 min, this and the first supernatant were pooled and brought to 50 ml with mannitol medium (without heparin). A sample of 1 ml was taken for protein estimation.

f) During the protein estimation the remaining 49 ml of mitochondrial fraction were diluted with mannitol medium (without heparin) and centrifuged at 10,000 g for 10 min.

g) The mitochondrial pellet was resuspended and brought to a final protein concentration of 2 mg/ml with mannitol medium (with heparin, 250 U/ml and 1% of serum albumin).

The entire procedure was carried out at 0-4°C and the centrifugations were performed in a swinging rotor (HB4) of a refrigerated Sorvall RC2-B centrifuge.

8. Protein estimation

Protein was estimated by the method of Lowry et al (1951). Bovine serum albumin was used as standard.

9. Counting of mitochondria

The different methods described of counting mitochondria based on the application of bacteriological counting chambers are not adequate due to the problem of counting barely visible and mobile mitochondria. Baranowicz (1972) therefore developed a method in which the mitochondrial suspension is mixed with an equal volume of red blood cell suspension of known cell concentrations and the resulting mixture used to prepare smears. The dry smear is observed under high magnification in the light microscope. The mean number of mitochondrial particles corresponding to one erythrocyte is then estimated from the smears.

This method has been adopted in the experimental work of

this thesis due to its simplicity and relatively short time requirement. The method was used as follows.

a) Preparation of erythrocyte suspension. At decapitation the blood of the rats was collected in a plastic beaker containing a few mgs of heparin and centrifuged for 10 min in a clinical centrifuge in order to eliminate plasma. The red blood cells were washed 3 times with mannitol medium (with heparin). The last sediment was resuspended in the mannitol medium and the erythrocytes counted in a Petroff-Hauser counting chamber. The final suspension contained usually from 6×10^9 to 9×10^9 erythrocytes per ml.

b) Preparation of smears. 1 ml of the erythrocyte suspension in mannitol medium and 1 ml of the mitochondrial suspension containing 2 mg protein/ml were thoroughly mixed in a plastic test tube and finally diluted with 2 ml of mannitol medium with heparin. A few smears were immediately prepared from the mixed suspension of mitochondria and erythrocytes.

c) Counting. Immediately after drying the smears on the slides the counting of mitochondria and erythrocytes was performed in 12 different fields under high magnification of a phase contrast microscope without immersion.

The standard error of the mean ratio obtained from counting the mitochondria and erythrocyte in the smears did not exceed 10% in relation to the final ratio of mitochondria to

erythrocytes. The number of mitochondria in 1 ml of the mitochondrial suspension may be calculated from the known number of erythrocytes added to the mitochondrial suspension, and from the established mean ratio of mitochondria to erythrocytes determined by counting both elements in the smears. Therefore as the amount of mitochondria and the amount of protein is known it is possible also to calculate the amount of protein of a single muscle mitochondrion.

10. Measurement of cytochrome oxidase activity

The 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 concentration of total and reduced cytochrome c was checked by the extinction coefficients reported by Yonetani (1965). The homogenates and mitochondrial fractions were previously rehomogenized for 1 min with Lubrol (0.3 mg/mg of protein) and diluted with the corresponding medium to the desired final protein concentration. These Lubrol-activated preparations were used for the measurement of cytochrome oxidase activity.

11. Measurement of NADH-Cyt c reductase (Total and Rotenone-insensitive)

NADH-cytochrome c reductase activity was measured spectrophotometrically at 30°C, by following the reduction of cytochrome c at 550nm . The assay mixture contained in 1 ml: 50 µmoles of phosphate buffer, pH 7.5; 0.1 µmoles of cytochrome c,

and 0.5 µmoles of NaCN (neutralized just before use). The enzyme preparation of 1 mg protein/ml was used in the assay in a volume equal to 100 µl. The reaction was started by adding 0.1 µmole of NADH. For the assay of the NADH-cytochrome c reductase (rotenone-insensitive) the assay medium also contained 3.5 mµmoles of rotenone. (Rotenone was stored at -20°C as a 1 mM stock solution in 95% ethanol). These assay methods are essentially those described by Hoppel and Cooper (1968). The concentration of total and oxidized cytochrome c was checked by the extinction coefficients reported by Yonetani (1965). The spectrophotometric assay of NADH-cytochrome c reductase (total and rotenone insensitive) were carried out in a Beckman DB spectrophotometer. The specific activities were determined from initial rates and were all proportional to enzyme concentration under the conditions described. The NADH-cytochrome c reductase (rotenone-sensitive) was estimated from subtracting the rotenone-insensitive activity from the total activity.

Additional information about the procedures and methods used in specific experiments are given in Chapter IV.

12) Statistical analysis of the results.

The statistical analysis of the results was performed according to Snedecor ("Statistical Methods", Ed. The Iowa State College Press, 1965).

CHAPTER IV: RESULTS

The following scheme has been adopted for the presentation of the results of each type of experiment:

a) Purpose of the experiment

b) Description of the experiment

c) Results and discussion

The presentation of the results will be followed by a general discussion and conclusions (Chapter V).

Section A. A COMBINED MORPHOLOGICAL AND BIOCHEMICAL STUDY OF MUSCLE MITOCHONDRIA IN WARM-ACCLIMATED AND COLD-ACCLIMATED RATS.

This section is divided into three parts:

1. Morphological study of muscle fibers and ultrastructural study of the mitochondria in both red and white muscle.
2. Estimation of cytochrome oxidase in red and white muscle from warm-acclimated and cold-acclimated rats.
3. Studies of mitochondria isolated from red and white muscle of warm-acclimated and cold-acclimated rats. These studies include the estimation of the number of mitochondria and the estimation of several biochemical parameters such as cytochrome oxidase activity and protein content of the isolated mitochondria.

1. Morphological study of muscle fibers and ultrastructural study of the mitochondria in both red and white muscle.

a) Purpose of the experiment: to see whether there are changes in the characteristics, proportions, distribution and ultrastructure of mitochondria of the 3 fiber types of skeletal muscle in the cold-acclimated rat.

b) Description of the experiment: 12 male rats were divided into equal groups of warm-acclimated and cold-acclimated rats. The rats had lived for approximately 1 month at their acclimation temperature when the experiment was started. Body weights of the rats at this time were, warm-acclimated: 327.20 ± 9.90 g (6 rats); cold-acclimated: 244.70 ± 8.50 g (6 rats).

The rats were killed by decapitation and the semitendinosus muscle removed from both legs. Samples for histological demonstration of succinic dehydrogenase and electron microscopy were taken and processed as described under Methods (Chapter III). Portions of red and white semitendinosus from cold-acclimated and warm-acclimated rats were stored separately in the deep freeze in order to use them in the quantitative estimation of cytochrome oxidase activity.

c) Results and discussion: Figure 27 shows a representative picture of a cross section of the semitendinosus muscle of warm-acclimated and cold-acclimated rats stained for succinic dehydrogenase activity. The muscle from the warm-acclimated rat shows the typical fiber distribution. The red portion consists primarily of fibers rich in mitochondrial enzymic activity, and the white portion consists almost entirely of fibers with low mitochondrial enzymic activity. These observations agree completely with those of Gauthier (1969 and 1970)

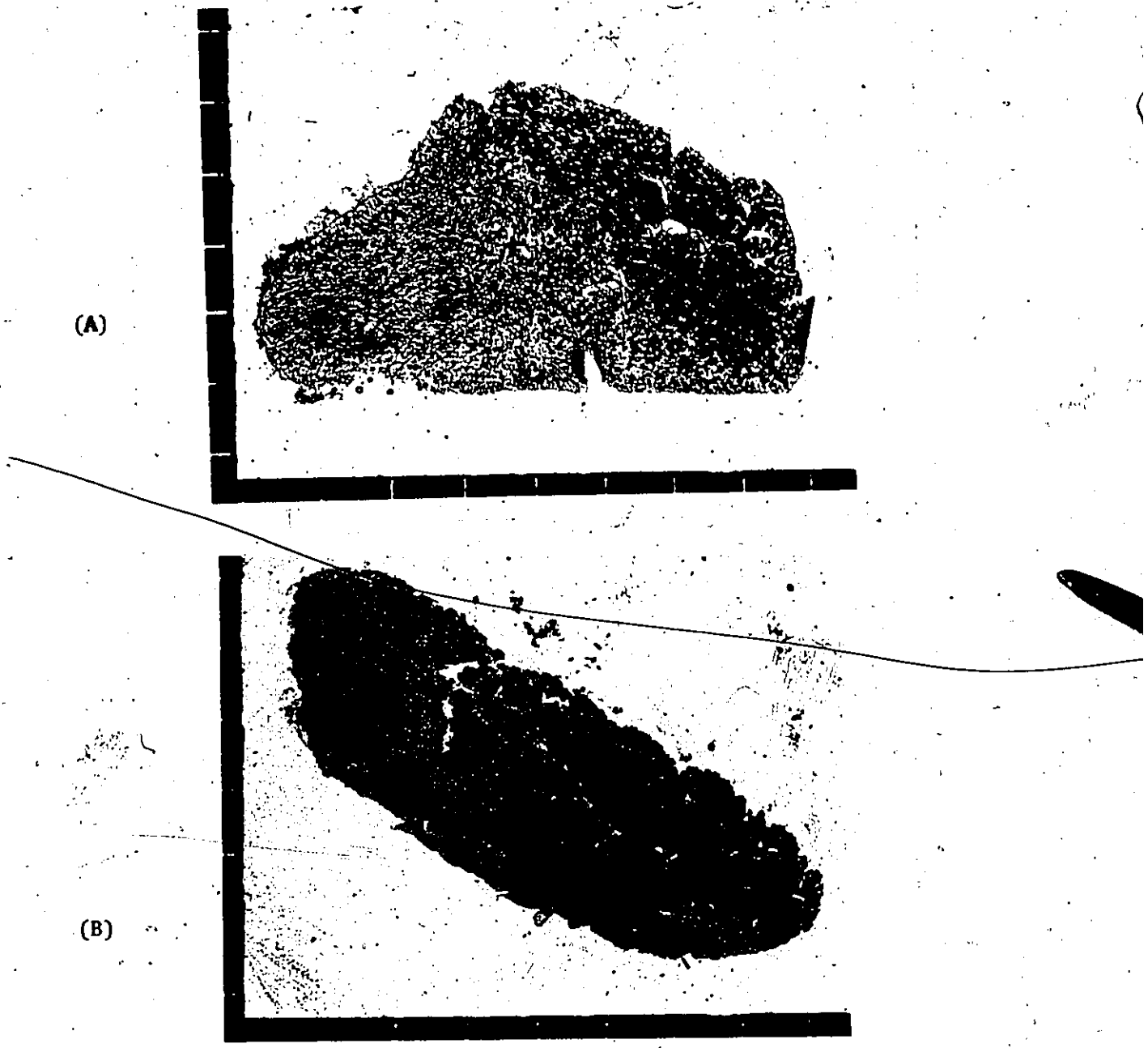


Figure 27. Transverse section through the entire width at center of the semitendinosus stained for succinic dehydrogenase.
(A) Muscle from Warm-acclimated rat;
(B) Muscle from Cold-acclimated rat.

(X 15)

for the same muscle. In contrast the muscle from the cold-acclimated rats presents a different aspect. The characteristics of the fibers and proportions of them seems to be altered. In both portions there is an apparent increase in the proportions of red fibers while the white fibers appear more like intermediate fibers. Observations of the sections in the light microscope at higher magnification revealed that alterations have taken place in the characteristics of the fibers in both red and white portions of the muscle of the cold-acclimated rats in comparison with the muscle from the warm-acclimated rats. A relative diminution in the sub-sarcolemmal aggregations of mitochondria of the red fiber was seen. Moreover, the white fiber also appeared to have an increased number of mitochondria because the normal fine network of mitochondria in the fiber was more densely stained giving the white fibers a general appearance more like that of intermediate fibers.

There was also an apparent decrease in the size of the fiber, as reported by Héroux (1958), but no increase in the number of them; in fact the counting of the total number of fibers in some sections showed no change in the total number of fibers in cold-acclimated rats compared with warm-acclimated rats. As the primary aim of this experiment was satisfied, in the sense that gross changes were observed in the fiber characteristics and proportions of them, no attempt was made to estimate the size and other morphological characteristics of the fibers. It was decided to study directly the ultra-

structure of the muscle mitochondria.

Figures 28, 29 and 30 show several electronmicrographs taken from both red and white fibers of the semitendinosus muscle of warm-acclimated and cold-acclimated rats. It should be indicated that the presented electronmicrographs were selected from 20 of them (for each group). The electronmicrographs most representative of the morphological characteristics of the groups under study were selected.

As can be seen in Figures 28A, 29A and 30A, in the red fiber from the control warm-acclimated rats, the subsarcolemmal aggregations of large, closely packed mitochondria are conspicuous and extensive. Cristae are abundant and consist, for the most part, of parallel arrays of fenestrated sheets. Similar large mitochondria are closely aligned to form longitudinal rows among myofibrils. Few, small and more or less elliptical profiles occur in pairs at the I bands. In the white fiber from the warm-acclimated rat (Fig. 28B, 29B and 30B) paired mitochondria at the I bands are the predominant form but there is only a small number of them. Subsarcolemmal aggregations of mitochondria are inconspicuous or absent, even in the nuclear region. When present they consist of a few slender mitochondria in which cristae are sparse. It should be noted that the characteristics of the red and white fibers of warm-acclimated rats correspond closely with those previously described by Gauthier (1969 and 1970). A direct comparison between the red and white fibers in the warm-acclimated rat shows that the glycogen content is apparently higher in the

red fiber, in agreement with previous observations (Peter et al, 1972; Gillespie et al, 1970; see Table 1).

As expected from the histological localization of succinic dehydrogenase the muscle fiber characteristics in the cold-acclimated rat present marked alterations in ultrastructure as can be seen in the figures 28, 29 and 30. In fact in the red fibers (Fig. 28C, 29C and 30C) the subsarcolemmal aggregations of large, closely packed mitochondria are less conspicuous and less extensive than in the warm rat. There is no apparent reduction of the cristae. The longitudinal rows of mitochondria among the myofibrils are also affected; the rows are narrower and the mitochondria appear more numerous. The most marked effect of the cold seems to be on the paired mitochondria at the I bands; these apparently increase in number, appearing more often and throughout all the fiber area. In the white fiber (Fig. 28D, 29D and 30D) the effects seem to be even more marked, giving to the fiber an ultrastructural appearance similar to the normal intermediate; no aggregations of mitochondria were observed, but the paired mitochondria at the I bands increased in number appearing very often and throughout the whole area of the fiber. Also it can be observed that the abundant granules of glycogen present in the fibers of the warm-acclimated rat are less conspicuous in the fibers of the cold-acclimated rats; this change can be observed very clearly in the red fiber.

In these experiments, a marked difference between mitochondria of muscle of cold-acclimated rats and mitochondria

Figure 28. Electron micrographs of semitendinosus muscle from:

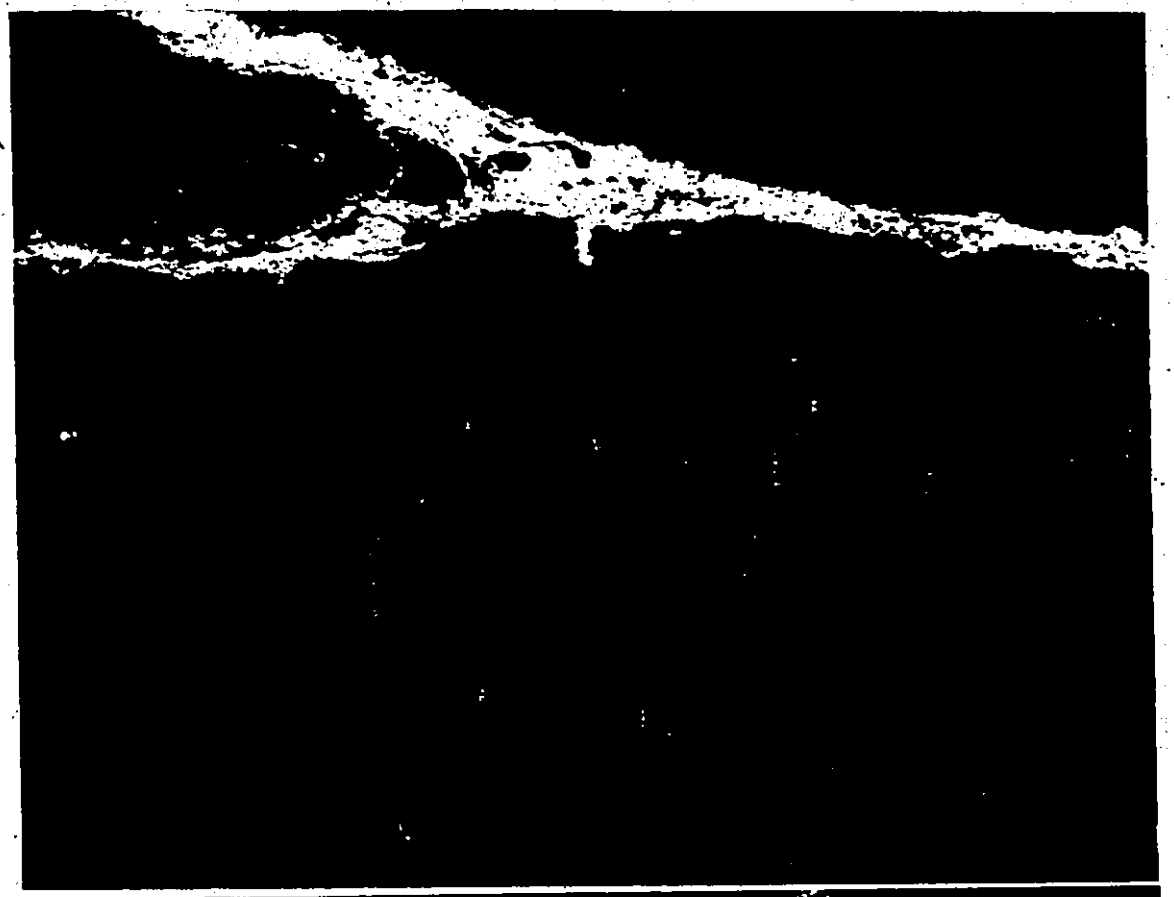
(A) Warm-acclimated rat,red fiber. Subsarcolemmal aggregations of large, closely packed mitochondria are conspicuous and extensive in this portion of the muscle.

(B) Warm-acclimated rat,white fiber. Subsarcolemmal aggregations of mitochondria are inconspicuous or absent.

(C) Cold-acclimated rat,red fiber. The subsarcolemmal aggregations of large,closely packed mitochondria are less conspicuous and less extensive than in the warm-acclimated rat. The most marked effect of the cold seems to be on the paired mitochondria at the I bands. These are smaller and more numerous.

(D) Cold-acclimated rat,white fiber. The effects of the cold seem to be even more marked in this type of fiber. The paired mitochondria at the I bands are increased in number appearing very often and throughout the whole area of the fiber.

Taken at 7000 X magnification with the electron microscope: magnification in printing X 2.



(A)

(B)

(C)

(D)

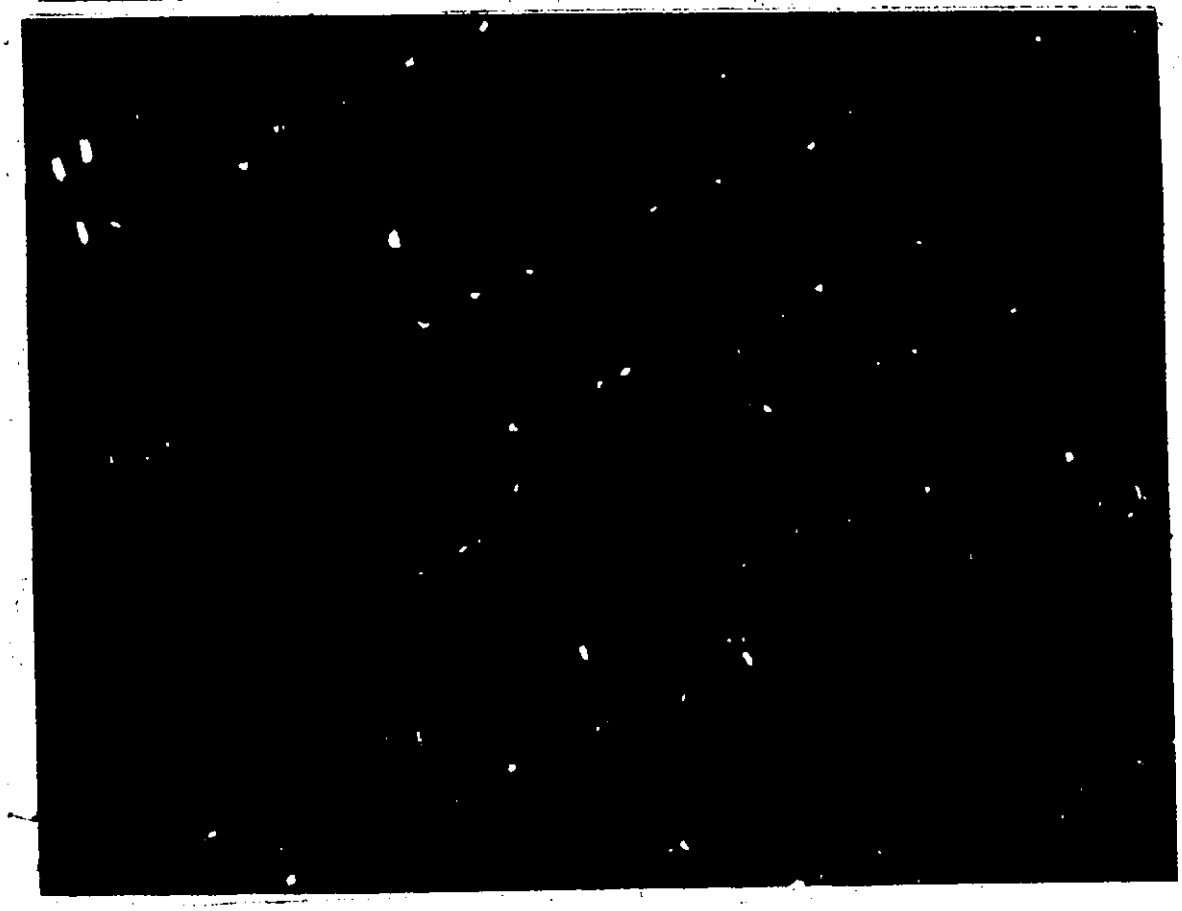


Figure 29. Electron micrographs of semitendinosus muscle from:

(A) Warm-acclimated rat,red fiber. Subsarcolemmal aggregations of large,closely packed mitochondria are conspicuous. Cristae are abundant and consist, for the most part,of parallel arrays of fenestrated sheets. Similar large mitochondria are closely aligned to form longitudinal rows among myofibrils.

(B) Warm-acclimated rat,white fiber. Subsarcolemmal aggregations of mitochondria are inconspicuous.

(C) Cold-acclimated rat,red fiber. The subsarcolemmal longitudinal rows of mitochondria are narrower than in the warm-acclimated rat.

(D) Cold-acclimated rat,white fiber. The effects of the cold seem to be even more marked in this type of fiber. The paired mitochondria at the I bands are increased in number appearing very often and throughout the whole area of the fiber.

Taken at 15000 X magnification with the electron microscope: magnification in printing X 2.

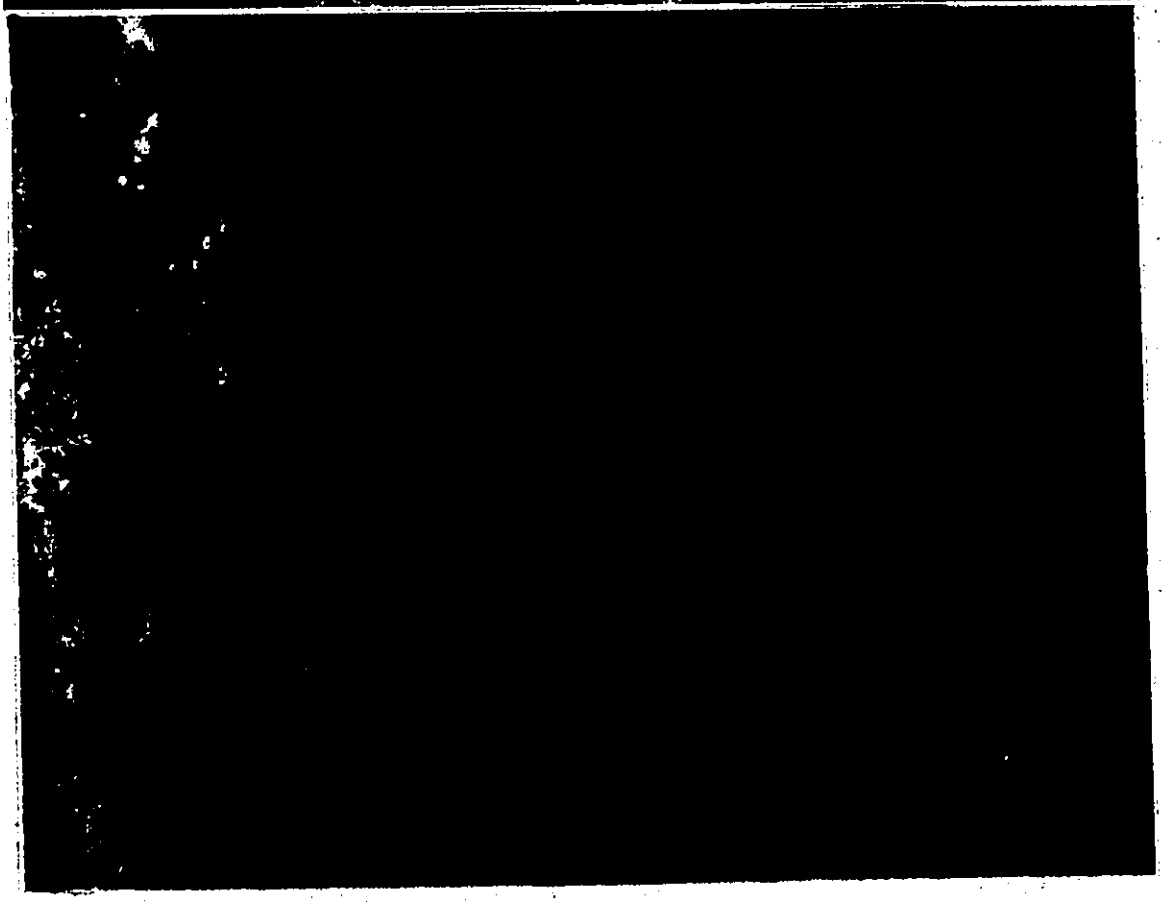
Figure 29. Electron micrographs of semitendinosus muscle from:

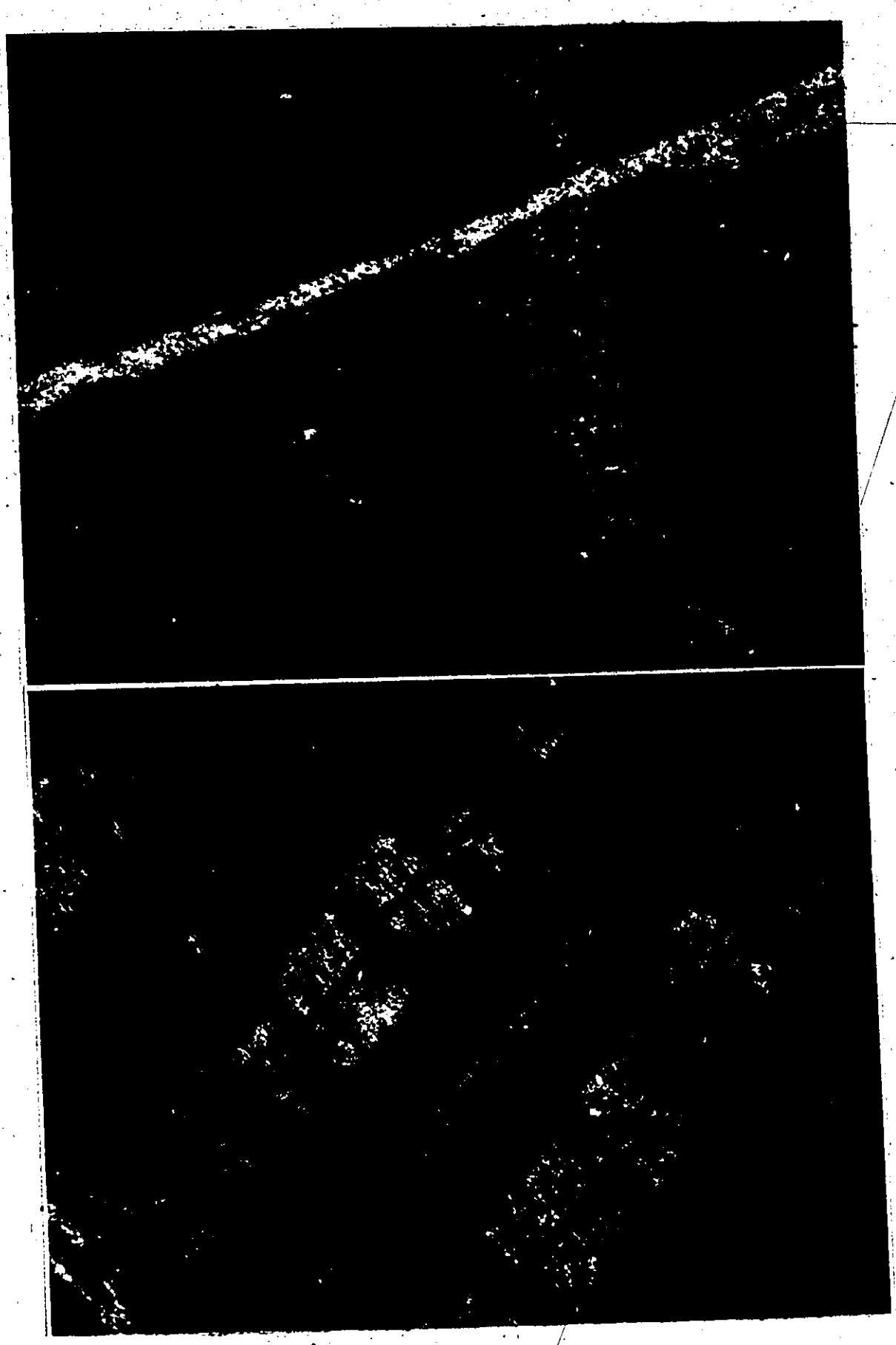
(A) Warm-acclimated rat, red fiber,
(B) Warm-acclimated rat, white fiber,
(C) Cold-acclimated rat, red fiber,
(D) Cold-acclimated rat, white fiber.

Taken at 15000 X magnification with the electron microscope: magnification in printing X 2.

(A)

(B)





(C)

(D)

Figure 30. Electron micrographs of semitendinosus muscle from:

(A) Warm-acclimated rat,red fiber,

(B) Warm-acclimated rat,white fiber,

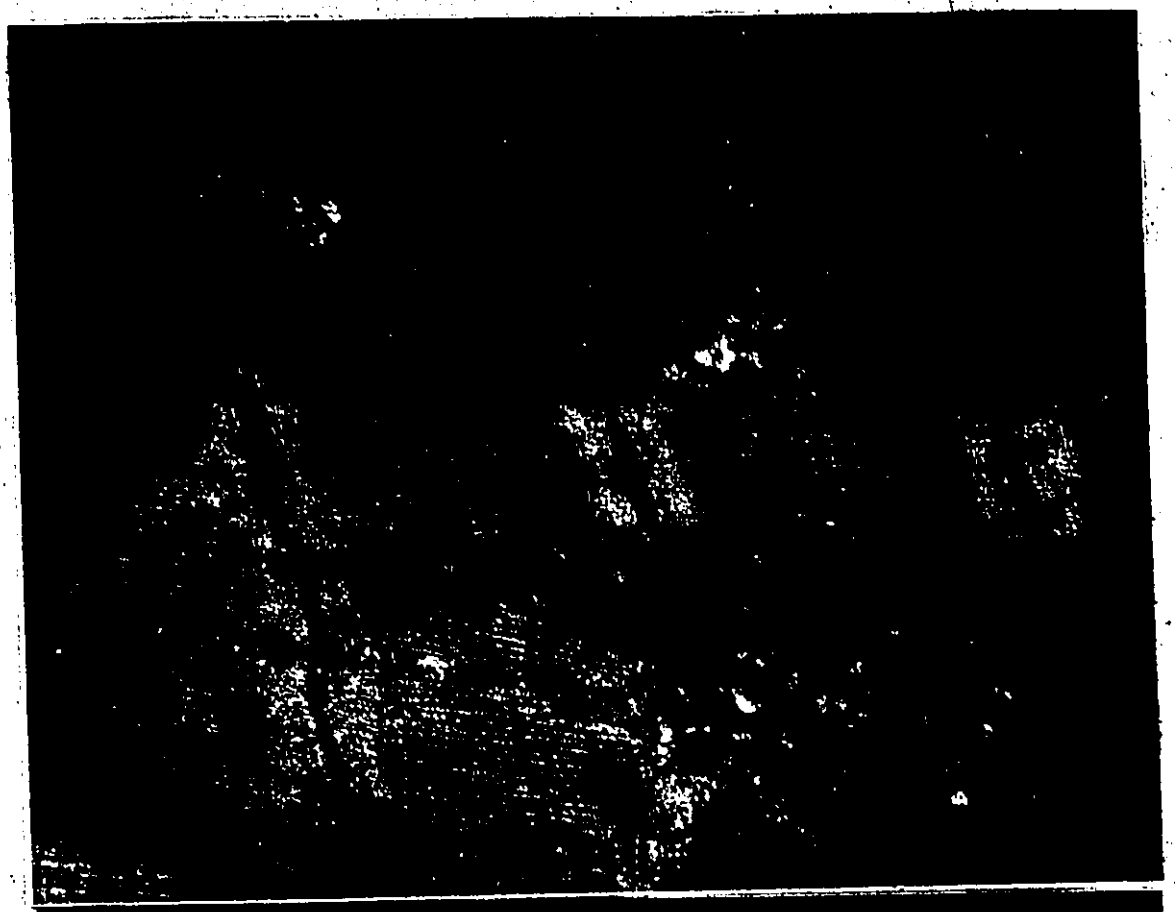
(C) Cold-acclimated rat,red fiber,

(D) Cold-acclimated rat,white fiber,

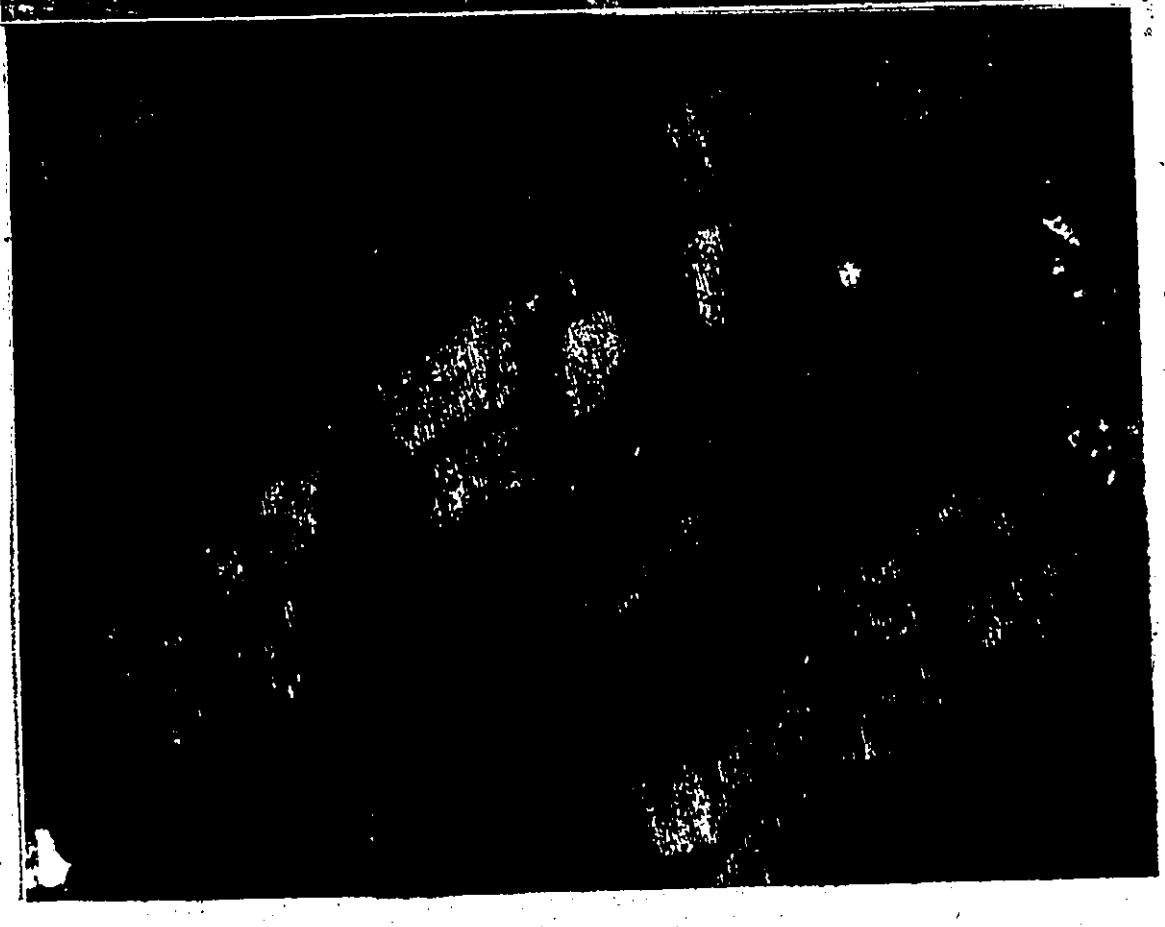
See legends to Figures 28 and 29 for description.

Taken at 20000 X magnification with the electron microscope: magnification in printing X 2.

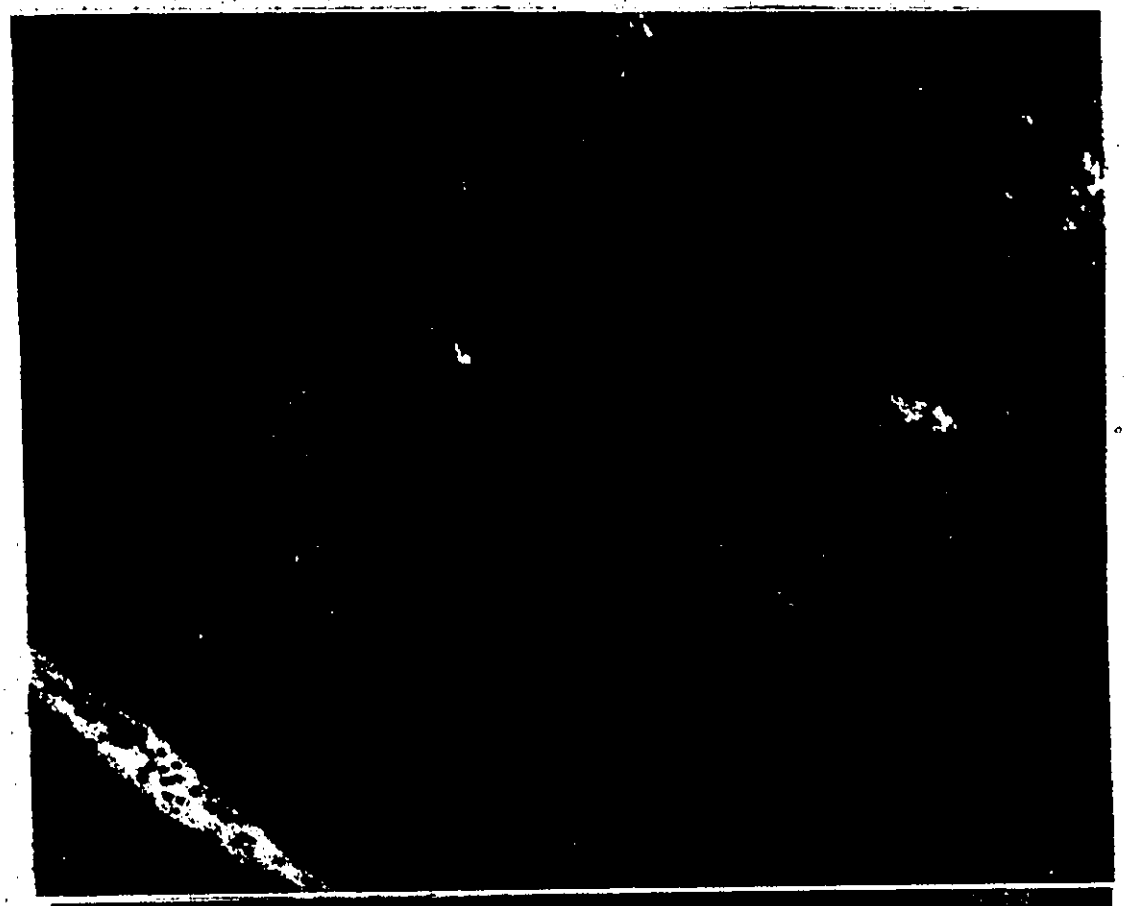
(A)



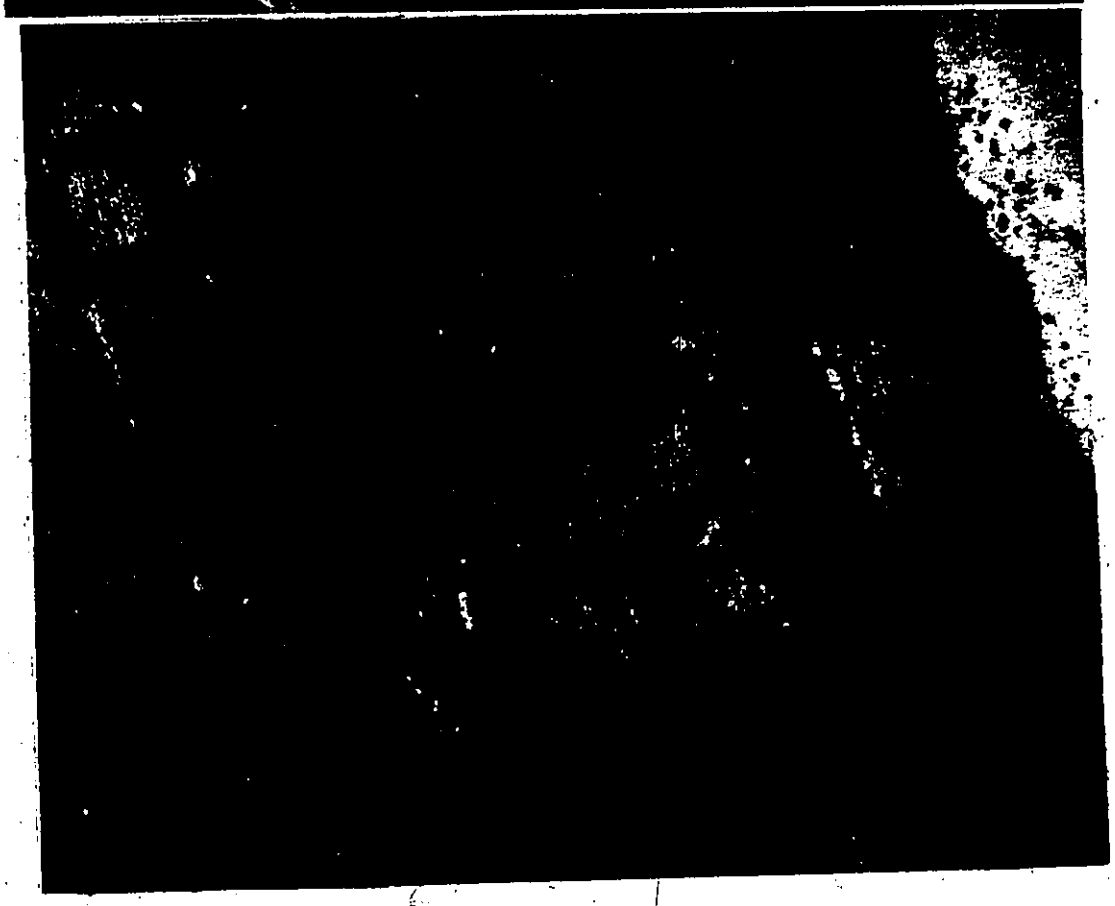
(B)



(C)



(D)



of warm-acclimated rats was seen. The histological and electron microscopic observations suggest that alterations occur in the fiber characteristics and that this is due principally to alterations in the mitochondrial population of the muscle from the cold-acclimated rats. The principal alterations seem to be an increase in the number and a reduction in the size of the mitochondria.

It should be noted that these general impressions are based upon the study of more than one hundred sections from different blocks of tissue from many different animals, not only the six which formed the groups in this experiment but also the control rats in the other experiments to be described in the other Sections. Nevertheless, they remain subjective impressions. It would be impossible to present in this thesis sufficient photographs to demonstrate clearly the appearance of the many different aspects of the red and white muscle fibers. No attempt was made to make a quantitative estimation of number and size of the mitochondria from the electron micrographs because an adequate method is not available which would permit such a measurement in skeletal muscle. Several stereological techniques provide a means for estimating morphological indices, but these techniques have been used mainly in describing intracellular measurements of cells with a random structure such as the hepatocyte (Loud, 1968; Weibel et al, 1969; Lang and Herbener, 1972); in a limited way they have been extended to the study of heart structure (Herbener et al, 1973; Laguens, 1971; Edington and Cosmas, 1972). However, the skeletal

muscle, because of its highly organized structure, does not permit the application of these methods.

Because of the subjective nature of the observations and because of the impossibility of presenting all the photographs taken or all the observations made with the electron microscope another method was sought which would permit a quantitative estimation of the number and size of mitochondria in muscle. Such a method was found (Baranowicz, 1972) and its use will be described in Part 3 of Section A.

With respect to the apparent reduced amount of glycogen, it seems possible that the increased activity of the sympathetic nervous system elicited by the cold could account for this observation in the muscle of the cold-acclimated rats (for reviews see Himms-Hagen, 1967 and 1972).

2. Estimation of cytochrome oxidase in red and white muscle from warm-acclimated and cold-acclimated rats.

a) Purpose of the experiment: As the electron microscopic observations indicated extensive alterations in the mitochondrial population in the muscle from the cold-acclimated rats but no apparent alterations in the cristae it was the purpose of this experiment to see from a biochemical measurement if the amount of mitochondrial inner membrane was altered. Cytochrome oxidase, an inner membrane enzyme (Ernster and Kuylenstierna, 1970), was selected for this purpose because the amount of this enzyme can be used as an index of the amount of mitochondrial inner membrane.

b) Description of the experiment: The frozen muscle (from the preceeding experiment) was minced and homogenized in 0.25 M sucrose in 0.01 M Tris buffer pH 7.4. The protein content of the homogenate was measured and portions of the homogenate were rehomogenized with Lubrol (0.3 mg/mg of protein) and diluted with 0.25 M sucrose in 0.01 M Tris buffer, pH 7.4, so that the final protein concentration was 1 mg/ml. This dilute, activated preparation was used for the measurement of cytochrome oxidase activity as described under Methods (Chapter III).

c) Results and discussion: Table VI shows that no increase in the specific activity of cytochrome oxidase was found in either the red or the white portions of the semitendinosus muscle from cold-acclimated rats in comparison with those from the warm-acclimated rats. Also it can be observed that the specific activity of cytochrome oxidase of the red muscle is more or less double that of the white muscle in both cold-acclimated and warm-acclimated rats. This is in close accord with the histological and electron microscopic observations of the mitochondrial content in both types of muscle.

The lack of increase in the specific activity of cytochrome oxidase and the electron microscopic observation that the amount of inner membrane of the muscle mitochondria has not changed indicate that there is no increase in oxidative capacity of skeletal muscle during cold-acclimation.

It seems, therefore, that the change that takes place in the muscle mitochondria under the stimulus of cold does not

TABLE VI. Specific activity of cytochrome oxidase in semitendinosus muscle homogenates (red and white) in warm-acclimated and cold-acclimated rats.

	Warm-acclimated	Cold-acclimated
Red portion	1.185 ± 0.134	1.091 ± 0.127
White portion	0.530 ± 0.038	0.499 ± 0.049

The activity is expressed as: µmoles cytochrome c oxidized/ mg. protein. min. Means ± SE. n = 6.

involve any alteration in total mitochondrial mass.

These results contrast with those of Hannon (1960) who reported increase in cytochrome oxidase activity in muscles from cold-acclimated rats.

3. Studies of isolated mitochondria from red and white muscle from warm-acclimated and cold-acclimated rats.

a) Purpose of the experiment: To study the changes in the mitochondria by a quantitative method which could give more reliable information about the number and size of the mitochondria without the subjective error inherent in the electron micrographic studies. As was explained above, because the structure of the skeletal muscle does not permit the use of stereologic techniques applied to the electron micrographs, it was decided to count the mitochondria in a suspension of isolated mitochondria using the method described by Baranowicz (1972), as described under Methods (Chapter III). Several biochemical parameters of these isolated mitochondria have also been studied.

b) Description of the experiment: As the semitendinosus is a relatively small muscle and the intention here was to isolate enough mitochondria in order to count them and perform several biochemical estimations a large number of rats was used. A total of 36 warm-acclimated (560.40 ± 4.90 g) and 29 cold-acclimated rats (404.90 ± 5.60 g) were used. Both groups of rats were living at their respective temperature of acclimation

for a period of approximately 4 months. Each group of rats was subdivided into 4 subgroups in order to repeat the experiment 4 times. The rats were killed and 5 g of each type of semitendinosus (red and white) were collected from both groups of rats. The mitochondria were isolated by the protease digestion procedure as described under Methods (Chapter III) (with half the amount of ATP, protease and total volume indicated). Cytochrome oxidase activity was estimated in both homogenate and final mitochondrial suspension in order to estimate the recovery in the suspension of isolated mitochondria of the total mitochondria present in the muscle.

c) Results and discussion: Table VII and Figure 31 show the data obtained in this experiment. A marked increase in the number of mitochondria per gram of muscle occurred in both red and white muscle of the cold-acclimated rats: the increase was larger in the red muscle. The size of the mitochondria, as indicated by the protein content per mitochondrion and the cytochrome oxidase activity per mitochondrion, was reduced in both red and white muscle. Thus the impression gained from the electron micrographs of a larger number of smaller mitochondria is confirmed by this more direct method. Despite the greater number of mitochondria per gram of muscle there is no change in the total mitochondrial mass of either red or white muscle of the cold-acclimated rat, as indicated by the similar cytochrome oxidase activities per gram of muscle and the similar mitochondrial protein content per gram of muscle in both red and white muscles. The specific activity of cytochrome

TABLE VII. Number of mitochondria and biochemical parameters of isolated mitochondria from red and white semitendinosus muscle from warm- and cold-acclimated rats.

	Red	White	P (red vs white)
Recovery:mg mitoch protein/g. muscle			
WARM-ACCLIMATED	2.97 ± 0.40	1.17 ± 0.06	<0.005
COLD-ACCLIMATED	2.18 ± 0.23	1.22 ± 0.13	<0.020
P (warm vs cold)	NS.	NS.	
Recovery:cytochrome oxidase, %			
WARM-ACCLIMATED	40.92 ± 2.68	26.95 ± 2.15	<0.010
COLD-ACCLIMATED	32.86 ± 3.04	28.74 ± 1.85	NS.
P(warm vs cold)	NS.	NS.	
Total mitochondrial protein/g. muscle			
WARM-ACCLIMATED	7.26 ± 0.75	4.38 ± 0.29	<0.020
COLD-ACCLIMATED	6.66 ± 0.51	4.35 ± 0.62	<0.050
P (warm vs cold)	NS.	NS.	
Protein:picograms/ mitochondrion			
WARM-ACCLIMATED	0.1107 ± 0.0050	0.1144 ± 0.0056	NS.
COLD-ACCLIMATED	0.0615 ± 0.0034	0.0732 ± 0.0068	NS.
P (warm vs cold)	<0.001	<0.005	

...continued

TABLE VII (continuation)

	Red	White	P (red vs white)
N°mitochondria/g. muscle (10^{-9})			
WARM-ACCLIMATED	65.297 ± 4.909	38.856 ± 4.307	<0.010
COLD-ACCLIMATED	108.838 ± 8.542	59.950 ± 8.294	<0.010
P (warm vs cold)	<0.005	<0.10*	
Specific activity cytochrome oxidase µatoms O_2/mg prot.min			
WARM-ACCLIMATED	18.124 ± 1.665	14.307 ± 0.628	<0.050
COLD-ACCLIMATED	19.135 ± 1.034	14.866 ± 1.307	<0.050
P (warm vs cold)	NS.	NS.	
N° mitochondria/mg. mitochondrial protein (10^{-9})			
WARM-ACCLIMATED	9.093 ± 0.405	8.806 ± 0.416	NS.
COLD-ACCLIMATED	16.410 ± 0.894	14.029 ± 1.303	NS.
P (warm vs cold)	<0.001	<0.010	
Cytochrome oxidase µatomsO_2/mitochondrion (10^{9})			
WARM-ACCLIMATED	1.983 ± 0.106	1.631 ± 0.077	<0.050
COLD-ACCLIMATED	1.169 ± 0.040	1.080 ± 0.122	NS.
P (warm vs cold)	<0.001	<0.010	
Cytochrome oxidase µatoms O_2/g. muscle.min.			
WARM-ACCLIMATED	128.153 ± 5.074	63.000 ± 6.751	<0.001
COLD-ACCLIMATED	126.399 ± 7.587	62.217 ± 4.554	<0.001
P (warm vs cold)	NS.	NS.	

Means ± SE. n = 4.

* Values up to 5% level were considered not significant, however, in this case it is important to indicate that the value is only slightly superior to the 5% level.

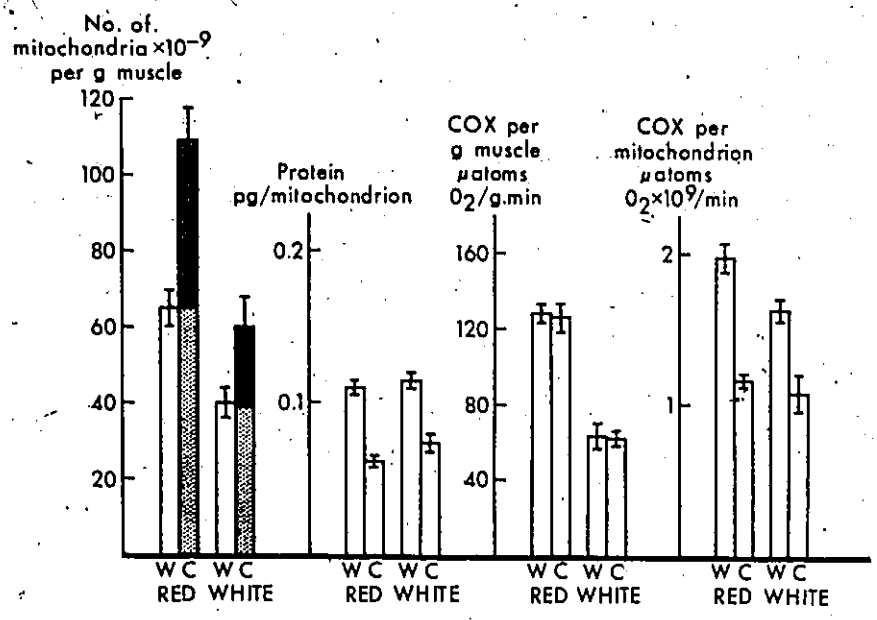


Figure 31. Number of mitochondria, protein content and cytochrome oxidase activity of isolated mitochondria from red and white semitendinosus muscle from warm-acclimated (W) and cold-acclimated (C) rats.

oxidase in the mitochondria is also unchanged by cold-acclimation.

As might have been expected the number of mitochondria per gram of muscle and the cytochrome oxidase activity per gram of muscle were greater in red muscle than in white muscle, in both warm-acclimated and cold-acclimated rats. The average size of the mitochondria, as indicated by protein content per mitochondrion, was similar in red and white muscles although the amount of cytochrome oxidase per mitochondrion and also the specific activity of cytochrome oxidase was slightly less in the mitochondria from white muscle.

As a summary of the experiments performed under the Section A it can be said that a marked difference between mitochondria of muscles of cold-acclimated rats and mitochondria of muscles of warm-acclimated control rats has been seen by using a combined approach of histological, ultrastructural and biochemical methods. This is the first time that such a difference has been observed.

The principal differences seen are:

i) a reduction in the size of the mitochondria but an increase in mitochondrial number. The mitochondrial mass per gram of muscle is unchanged but the way in which it is distributed is altered.

ii) a reduction in the size of the muscle fibers. The distribution of fibers changes after cold-acclimation. White fibers appear to be replaced by intermediate-type fibers.

Unlike other adaptive changes in muscle mitochondria (see Section H, Introduction), there appears to be no increase in the inner membrane, as indicated by the lack of effect of cold-acclimation on the ultrastructure of the cristae. In spite of the apparent increase in the number of red fibers the changes in muscle mitochondria that occur after prolonged exposure to cold do not involve a major increase in oxidative capacity. Edington and Cosmas (1972), observing the effect of training on the mitochondrial size distributions in rat heart, found also a shift in the direction of smaller mitochondria. They suggested the existence of a mechanism which increases the surface to volume ratio in response to training which might possibly be an indication of increased physiological function.

As the described changes that take place in muscle mitochondria after cold-acclimation differ markedly from those described as a consequence of other stimuli which change muscle function it is suggested that the changes observed in cold-acclimation are due to the development of nonshivering thermogenesis rather than to the initial period of shivering, however the experiments so far described do not allow one to distinguish between the two possibilities.

The changes observed in muscle mitochondria of cold-acclimated rats are different from the changes known to occur in brown adipose tissue of cold-acclimated rats. The brown adipose tissue grows and at the same time acquires more and larger mitochondria which have an increased number of cristae and a higher specific activity of respiratory enzymes (Suter,

1969; Skala et al, 1970; Barnard, 1969; Lindgren and Barnard, 1972). Muscle, on the other hand, does not grow. Although it acquires more mitochondria, these mitochondria are smaller and have an unchanged specific activity of respiratory enzymes. The total mitochondrial mass is increased in brown adipose tissue whereas in muscle it is unchanged. The lack of significant changes in the ultrastructure of liver mitochondria (Kim, 1967) during cold-acclimation suggest that adaptive changes take place only in those organs where nonshivering thermogenesis occurs (namely, muscle and brown adipose tissue).

The differences between muscle and brown adipose tissue in the adaptive changes induced by cold-acclimation in their mitochondria suggest that the mechanism of nonshivering thermogenesis may be different in these two tissues. It is not known in what way the changes observed in the muscle mitochondria might contribute to or control the mechanism of nonshivering thermogenesis.

Section B. USE OF OXYTETRACYCLINE TO PREVENT THE DEVELOPMENT OF THE ADAPTATION FOR NONSHIVERING THERMOGENESIS AND TO REVERSE THE ADAPTATION AFTER ITS DEVELOPMENT: CORRELATION WITH BIOCHEMICAL PROPERTIES OF BROWN ADIPOSE TISSUE, MUSCLE AND LIVER.

a) Purpose of the experiment: It was indicated in the Introduction that inhibition of mitochondrial protein synthesis with oxytetracycline inhibits the development of the enhanced response to noradrenaline induced by cold-acclimation (Himms-

Hagen, 1971). These experiments were designed to find out whether inhibition of mitochondrial protein synthesis with oxytetracycline can reverse the enhanced response to noradrenaline when it has already developed during cold-acclimation and whether the inhibition by treatment with oxytetracycline of the development of the enhanced response during cold-acclimation is reversible when the treatment is stopped. These experiments are preliminary to those described in Sections C and D in which the effect of oxytetracycline on the increased number of mitochondria in muscle of cold-acclimated rats will be described.

b) Description of the experiment: 63 male rats weighing 80-90 g were kept for 5-9 days in groups in large cages at room temperature (25-28°C). They were then divided into two groups; Group S: 154.67 ± 2.18 g (30 rats) and Group T: 168.48 ± 1.89 g (33 rats). Rats of Group T were injected intramuscularly in both legs with oxytetracycline twice a day as indicated in Methods. The total dose per day was 200 mg/kg during the first week; for the rest of the experiment the dose was increased to 300 mg/kg. Some of the rats from Groups S and T (5 rats from each group) received an infusion of noradrenaline on the day of the first injection; these rats are referred to as zero time rats. The remainder of the rats from Groups S and T were placed in individual metal cages in the cold room (4°C). After these rats had been in the cold continuously for 2 weeks, receiving injections twice daily, they were transferred to room tempera-

ture (5 rats from Group S and 5 rats from Group T) after a 9 p.m. injection. They were reinjected at 9 a.m. the following morning and infused with noradrenaline on the same day; these rats are referred to as week 2 rats. The remainder of the rats from Groups S and T were maintained in the cold. At week 2 for some of the rats from group T (10 rats) the treatment with oxytetracycline was suspended and replaced by saline injections (this group is referred to as T-S). Some of the rats from the Group S (10 rats) were treated at this time with oxytetracycline (300 mg/kg); this group is referred to as S-T. Subsequently the rats from the four groups (S, T, S-T and T-S) remained in the cold for a third week. At the end of the week 3, 5 rats from each of the four groups were transferred to room temperature after a 9 p.m. injection and treated as described for those of week 2. The remainder of the rats of the four groups were maintained in the cold for another week (week 4) at the end of which all were transferred to room temperature after a 9 p.m. injection and processed as described previously.

In all cases immediately after the noradrenaline infusion the rats were killed, the organs removed, weighed and stored frozen. Later they were treated as was indicated in Methods (Chapter III).

c) Results and discussion: Table VIII and Figure 32 show the calorigenic response to noradrenaline. The expected increase in the calorigenic response to noradrenaline was observed in the saline-treated rats (Group S) after 2 weeks in the cold, remaining at this high level at week 3 and 4; however,

TABLE VIII. Total increase in oxygen uptake during a 30 min intravenous infusion of noradrenaline before and after 2, 3 and 4 weeks of cold exposure with and without oxytetracycline treatment.

	ml O_2/100 cm^2.30 min.
Week 0.	
Group S* (n=5)	15.10 ± 1.80
Group T* (n=5)	18.44 ± 3.70
P (S vs T)	NS.
Week 2.	
Group S (n=4)	55.03 ± 5.70
Group T (n=3)	28.70 ± 3.10
P (S vs T)	<0.020
Week 3.	
Group S (n=5)	50.72 ± 1.24
Group T (n=4)	30.34 ± 5.20
Group S-T* (n=5)	41.24 ± 2.46
Group T-S* (n=4)	58.00 ± 3.70
P (S vs T)	<0.005
P (S vs S-T)	<0.010
P (S vs T-S)	NS.
Week 4	
Group S (n=5)	55.20 ± 2.60
Group T (n=5)	28.70 ± 2.23
Group S-T (n=4)	39.20 ± 1.37
Group T-S (n=4)	65.90 ± 0.60
P (S vs T)	<0.001
P (S vs S-T)	<0.005
P (S vs T-S)	<0.010

Means ± SE.

* The following symbols are used: S: rats injected daily with saline for the time indicated; T: rats injected daily with oxytetracycline for the time indicated; S-T: rats injected daily with saline for two weeks, then daily with oxytetracycline for an additional one (week 3) or two (week 4) weeks; T-S: rats injected daily with oxytetracycline for two weeks, then daily with saline for an additional one (week 3) or two (week 4) weeks. These same symbols are used in Tables IX - XVII. For further details see text.

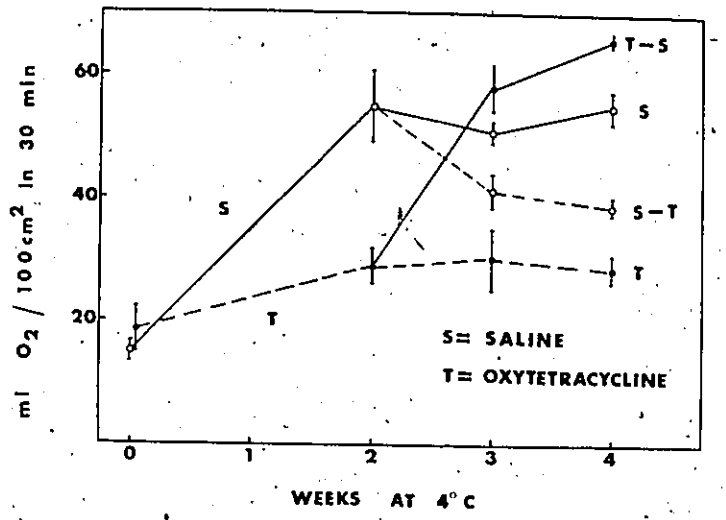


Figure 32. Effect of oxytetracycline administration during acclimation to cold on the calorigenic response to noradrenaline . During the first 2 weeks,rats were injected twice daily either with saline or oxytetracycline. The oxytetracycline treatment was continued for the remaining 2 weeks in some of the oxytetracycline-treated rats (T). During these last 2 weeks the dose of oxytetracycline was increased by 50%. Other rats which had been treated with oxytetracycline for the first 2 weeks were treated with saline for the last 2 weeks (T-S) . Some of the rats that were treated with saline for the first 2 weeks were further treated with saline during the last 2 weeks (S); other saline-treated rats were treated with oxytetracycline during the last 2 weeks (S-T).

the increase was very much smaller in the oxytetracycline-treated rats (Group T). Thus, treatment with oxytetracycline inhibits the development of the enhanced calorigenic response to noradrenaline during 2 or more weeks of adaptation to cold. This result is in accord with those of Himms-Hagen (1971). When the treatment with oxytetracycline was started after the development of the enhanced calorigenic response had occurred a marked reduction in the metabolic response was observed, indicating that the maintenance of the adaptive state is also inhibited by the treatment with the antibiotic (Group S-T at week 3 and 4). On the other hand, if the treatment with the antibiotic was suspended after 2 weeks the rats exhibited a rapid increase in the metabolic response to noradrenaline within one week after stopping the treatment (Group T-S), indicating that the inhibition of the development of the metabolic response to noradrenaline is readily reversible.

The treatment with oxytetracycline in rats undergoing cold-acclimation had no significant effect on the growth of the rats at week 2. In fact only a slight decrease in body weight was observed, which was not statistically significant (Table IX). However, if the treatment is continued for 2 extra weeks (weeks 3 and 4) the rats stop growing normally and in some cases they lose weight and die. It should be indicated here that the experiment was designed in order to have 5 rats for groups, but as can be seen in all the tables of this section, this is not the case because some rats died during the period of acclimation or during the noradrenaline infusion. Therefore, in the

TABLE IX. Body weights (g) of rats after 2, 3 and 4 weeks of cold exposure with or without oxytetracycline treatment.

WEEK 0.	
Group S* (n=5)	148.00 ± 6.33
Group T (n=5)	159.00 ± 2.92
P (S vs T)	NS.
WEEK 2.	
Group S (n=4)	225.00 ± 6.49
Group T (n=3)	197.30 ± 13.50
P (S vs T)	NS.
WEEK 3.	
Group S (n=5)	238.40 ± 7.74
Group T (n=4)	175.25 ± 13.26
Group S-T (n=5)	195.00 ± 13.26
Group T-S (n=4)	234.25 ± 10.90
P (S vs T)	<0.005
P (S vs S-T)	<0.025
P (S vs T-S)	NS.
WEEK 4.	
Group S (n=5)	278.80 ± 11.87
Group T (n=5)	187.80 ± 6.12
Group S-T (n=4)	209.80 ± 11.70
Group T-S (n=4)	285.80 ± 8.45
P (S vs T)	<0.001
P (S vs S-T)	<0.005
P (S vs T-S)	NS.

Means ± SE.

* Symbols are the same as in Table VIII

TABLE X. Wet weight and protein content of interscapular brown adipose tissue before and after 2, 3 and 4 weeks of cold-exposure with or without treatment of oxytetracycline.

Groups:	S*	T	S-T	T-S
WEEK 0.				
n	5	5		
Wet weight,g	0.198 ± 0.005	0.260 ± 0.041		
P (S vs other)		NS.		
Protein, mg/mg	0.131 ± 0.036	0.097 ± 0.009		
P(S vs other)		NS.		
Protein,total mg.	25.80 ± 7.18	24.00 ± 2.47		
P(S vs other)		NS.		
WEEK 2.				
n	4	3		
Wet weight,g	0.321 ± 0.020	0.320 ± 0.031		
P(S vs other)		NS.		
Protein,mg/mg	0.179 ± 0.024	0.177 ± 0.015		
P(S vs other)		NS.		
Protein,total mg.	56.25 ± 5.50	56.33 ± 5.79		
P(S vs other)		NS.		
WEEK 3.				
n	5	4	5	4
Wet weight,g	0.330 ± 0.018	0.347 ± 0.031	0.308 ± 0.021	0.453 ± 0.030
P(S vs other)		NS.	NS.	<0.01
Protein,mg/mg	0.181 ± 0.012	0.176 ± 0.018	0.221 ± 0.035	0.154 ± 0.014
P(S vs other)		NS.	NS.	NS.
Protein,total mg.	59.60 ± 4.68	60.50 ± 6.70	66.40 ± 2.24	68.75 ± 4.11
P(S vs other)		NS.	NS.	NS.
WEEK 4.				
n	5	5	4	4
Wet weight,g	0.416 ± 0.027	0.326 ± 0.011	0.365 ± 0.026	0.550 ± 0.047
P(S vs other)		<0.020	NS.	NS.
Protein,mg/mg	0.174 ± 0.007	0.177 ± 0.011	0.168 ± 0.010	0.162 ± 0.006
P(S vs other)		NS.	NS.	NS.
Protein,total mg.	71.80 ± 3.06	57.20 ± 2.08	61.50 ± 6.38	89.00 ± 8.50
P(S vs other)		<0.005	NS.	NS.

* Symbols are the same as in Table VIII

TABLE XI. Wet weight and protein content of muscle† before and after 2, 3 and 4 weeks of cold-exposure with or without oxytetracycline treatment.

Groups:	S*	T	S-T	T-S
WEEK 0.				
Wet weight,g	67.28 ± 2.89	72.24 ± 1.33		
P(S vs other)		NS.		
Protein,mg/mg	0.170 ± 0.012	0.175 ± 0.003		
P(S vs other)		NS.		
Protein,total mg.	11522 ± 1110	12652 ± 449		
P(S vs other)		NS.		
WEEK 2.				
Wet weight,g	97.55 ± 2.87	85.60 ± 5.87		
P(S vs other)		NS.		
Protein,mg/mg	0.181 ± 0.004	0.196 ± 0.013		
P(S vs other)		NS.		
Protein,total mg.	17611 ± 406	16848 ± 1725		
P(S vs other)		NS.		
WEEK 3.				
Wet weight, g	103.38 ± 3.33	76.03 ± 5.78	84.62 ± 5.89	101.60 ± 4.74
P(S vs other)		<0.005	<0.025	NS.
Protein,mg/mg	0.180 ± 0.004	0.185 ± 0.006	0.186 ± 0.007	0.170 ± 0.004
P(S vs other)		NS.	NS.	NS.
Protein,total mg.	18612 ± 247	13949 ± 672	15890 ± 1618	17261 ± 575
P(S vs other)		<0.005	NS.	NS.
WEEK 4.				
Wet weight,g	120.90 ± 5.11	81.30 ± 2.70	91.05 ± 5.10	123.00 ± 3.70
P(S vs other)		<0.001	<0.005	NS.
Protein,mg/mg	0.183 ± 0.003	0.189 ± 0.007	0.175 ± 0.007	0.163 ± 0.009
P(S vs other)		NS.	NS.	NS.
Protein, total mg.	22062 ± 724	15436 ± 1087	15959 ± 1218	20463 ± 1553
P(S vs other)		<0.001	<0.005	NS.

Means ± SE. * Symbols are the same as in Table VIII.

† For the calculation of total muscle weight and total protein it is assumed that muscle represents 43.3% of body weight (Heroux, 1958).

TABLE XII. Wet weight and protein content of liver before and after 2, 3 and 4 weeks of cold-exposure with or without oxytetracycline treatment.

Groups:	S*	T	S-T	T-S
WEEK 0.				
Wet weight, g.	5.50 ± 0.20	5.85 ± 0.12		
P(S vs other)		NS.		
Protein,mg/mg.	0.213 ± 0.008	0.226 ± 0.013		
P(S vs other)		NS.		
Protein, total mg.	1168.8 ± 45.9	1319.8 ± 60.6		
P(S vs other)		NS.		
WEEK 2.				
Wet weight, g.	9.44 ± 0.40	9.32 ± 0.82		
P(S vs other)		NS.		
Protein,mg/mg.	0.227 ± 0.008	0.191 ± 0.008		
P(S vs other)		NS.		
Protein, total mg.	2140.6 ± 100.9	1770.8 ± 137.9		
P(S vs other)		NS.		
WEEK 3.				
Wet weight,g.	9.54 ± 0.51	8.13 ± 1.02	8.32 ± 0.57	10.50 ± 0.65
P(S vs other)		NS.	NS.	NS.
Protein,mg/mg.	0.203 ± 0.008	0.189 ± 0.009	0.200 ± 0.010	0.195 ± 0.005
P(S vs other)		NS.	NS.	NS.
Protein, total mg.	1937.5 ± 119.8	1531.3 ± 178.2	1656.3 ± 116.5	2039.1 ± 107.8
P(S vs other)		NS.	NS.	NS.
WEEK 4.				
Wet weight,g.	12.26 ± 0.69	8.71 ± 0.34	9.61 ± 0.96	12.77 ± 0.58
P(S vs other)		<0.005	NS.	NS.
Protein,mg/mg.	0.198 ± 0.004	0.201 ± 0.006	0.213 ± 0.017	0.194 ± 0.005
P(S vs other)		NS.	NS.	NS.
Protein, total mg.	2425.0 ± 118.0	1756.3 ± 86.4	2023.4 ± 169.9	2468.8 ± 62.5
P(S vs other)		<0.005	<0.010	NS.

Means ± SE.

*Symbols are the same as in Table VIII

tables are indicated only the number of rats which survived all the stages of the experiment. Table IX also shows that when partially cold-acclimated rats (Group S, week 2) were treated with oxytetracycline for one or two weeks (Group S-T; week 3 and 4) the rate of growth decreased. On the other hand, when the treatment with the antibiotic was suspended at week 2 (Group T, week 2) and replaced by saline injections the somewhat lower rate of growth of these rats quickly increased and equalized the rate of growth of the Group S (Group T-S; week 3 and 4).

Like the body weight, the weight and protein content of tissues under study (interscapular brown adipose tissue, see Table X; skeletal muscle, see Table XI; and liver, see Table XII) were not significantly affected after two weeks of treatment; only after 3 or 4 weeks some inhibitory effects of the antibiotic can be seen. In all cases no significant differences were found in the amount of protein per unit weight of tissue. The usual and marked increase in wet weight and total protein of brown adipose tissue occurred in response to cold in both saline-treated and oxytetracycline-treated rats (Table X). Muscle and liver growth was more or less unaffected until week 3. It seems therefore that the treatment with oxytetracycline does not affect substantially the cytoplasmic protein synthesis in the organs under study.

On the other hand the mitochondrial protein synthesis of brown adipose tissue was altered significantly by the treatment with oxytetracycline during the cold-acclimation. Figure 33

and Tables XIII and XIV show that a large increase in specific activity of cytochrome oxidase occurred after two weeks of cold-acclimation (Group S); the specific activity remained high during weeks 3 and 4 (in both homogenate and isolated mitochondria). No such increase in the specific activity of cytochrome oxidase was seen in the interscapular brown adipose tissue of the oxytetracycline-treated rats (Group T). It should be noted that when the partially cold-acclimated rats (Group S, week 2) were treated with the antibiotic for 1 or 2 weeks (Group S-T, weeks 3 and 4) the specific activity of cytochrome oxidase was markedly reduced. In contrast, when the treatment was suspended (Group T-S, weeks 3 and 4) the enzyme increased in activity (see Fig. 33 and Tables XII and XIV). Thus, although the growth of the interscapular brown adipose tissue in response to exposure to cold is not altered by oxytetracycline, the increase in amount of a specific mitochondrial inner membrane enzyme, cytochrome oxidase is inhibited by the antibiotic.

Another change in the brown adipose tissue, the increase in specific activity of rotenone-sensitive NADH-cytochrome c reductase (Sottocasa et al, 1967), another inner membrane enzyme, was also prevented by the antibiotic (Table XIV). As in the case of cytochrome oxidase, the inhibition was observed when the treatment with oxytetracycline was started after the rats were partially cold-acclimated and the inhibition was reversed when the antibiotic treatment was suspended. Table XIV also shows the increase in the specific activity of rotenone-

insensitive NADH-cytochrome c reductase, an outer membrane enzyme (Sottocasa et al, 1967), which was not significantly inhibited by the oxytetracycline treatment. The lack of inhibition of this enzyme could be expected, due to the fact that the outer membrane enzymes are made on the cytoribosomes and the mitochondrial protein synthesis has nothing to do with its synthesis (for reviews see Rabinowitz and Swift, 1970; Ashwell and Work, 1970; Beattie, 1971).

The total amount of cytochrome oxidase in interscapular brown adipose tissue (Table XIII) was increased more than four times by weeks 2-3, and almost 6 times by week 4 of cold-acclimation in the saline-treated rats. This increase was due to increases in both the total amount of tissue and the amount of enzyme per unit of weight of tissue. This increase in total activity of cytochrome oxidase was markedly inhibited at week 2, 3 and 4 in the oxytetracycline-treated rats. The smaller increase in total activity observed at week 3 and 4 is probably due to the increase in size of the tissue.

From these experiments it can be concluded that although the growth of the interscapular brown adipose tissue during cold-acclimation is not altered by oxytetracycline, the increase in amount of inner mitochondrial membrane, as indicated by the increases in cytochrome oxidase and rotenone-sensitive NADH-cytochrome c reductase, is prevented, presumably by the action of the antibiotic to inhibit mitochondrial protein synthesis. The inhibitory effect is readily reversible. Moreover, if the inhibition is started only after the increase in

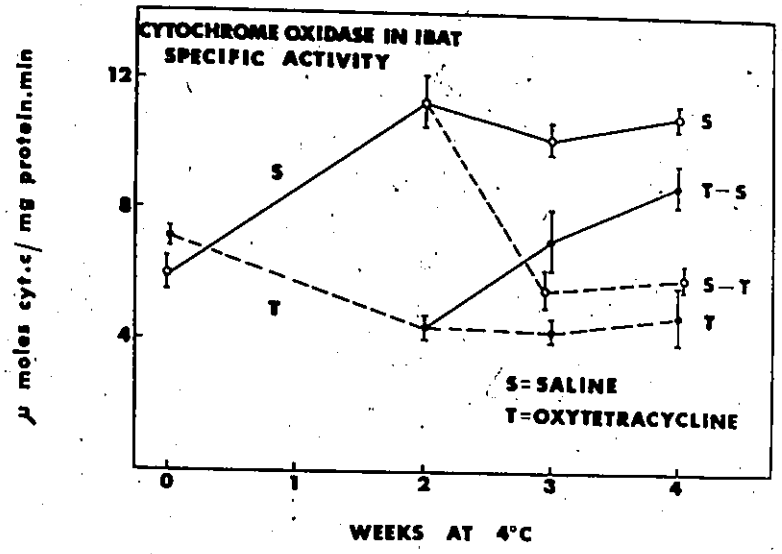


Figure 33. Effect of oxytetracycline on the specific activity of cytochrome oxidase of interscapular brown adipose tissue (IBAT) during 4 weeks of cold exposure. For further details see legend to Fig.32.

TABLE XIII. Specific activity and total cytochrome oxidase activity in homogenate of interscapular brown adipose tissue before and after 2,3 and 4 weeks of cold-exposure with or without treatment by oxytetracycline.

	Specific activity†	Total activity†
WEEK 0.		
Group S*(n=5)	5.94 ± 0.54	139.96 ± 25.08
Group T(n=5)	7.16 ± 0.36	168.56 ± 9.58
P(S vs T)	NS.	NS.
WEEK 2.		
Group S(n=4)	11.28 ± 0.82	622.66 ± 29.72
Group T(n=3)	4.44 ± 0.40	153.56 ± 42.02
P(S vs T)	<0.005	<0.005
WEEK 3.		
Group S(n=5)	10.20 ± 0.50	614.86 ± 71.58
Group T(n=4)	4.32 ± 0.42	263.02 ± 48.08
Group S-T(n=5)	5.56 ± 0.56	357.64 ± 39.36
Group T-S(n=4)	7.10 ± 0.94	484.44 ± 64.68
P(S vs T)	<0.001	<0.001
P(S vs S-T)	<0.001	<0.001
P(S vs T-S)	<0.050	<0.050
WEEK 4.		
Group S(n=5)	10.88 ± 0.42	781.80 ± 45.52
Group T(n=5)	4.78 ± 0.90	275.18 ± 37.78
Group S-T(n=4)	5.96 ± 0.42	373.98 ± 64.74
Group T-S(n=4)	8.78 ± 0.66	769.94 ± 50.10
P(S vs T)	<0.001	<0.001
P(S vs S-T)	<0.001	<0.001
P(S vs T-S)	<0.050	NS.

Means ± SE.

†Specific activity: µmoles cytochrome c oxidized/mg protein.min.
Total: µmoles cytochrome c oxidized per min by total organ.

*Symbols are the same as in Table VIII.

TABLE XIV. Specific activity of cytochrome oxidase, NADH-cytochrome c reductase (Rotenone sensitive and insensitive) in interscapular brown adipose tissue mitochondria before and after 2, 3 and 4 weeks of cold exposure with or without oxytetracycline treatment.

	Cytochrome† Oxidase	NADH-cyt c Red.† Rot.insensitive	NADH-cyt c Red.† Rot. sensitive.
WEEK 0.			
Group S*(n=5)	9.76 ± 0.94	0.018 ± 0.001	0.140 ± 0.015
Group T(n=5)	9.30 ± 0.56	0.017 ± 0.003	0.119 ± 0.014
P(S vs T)	NS.	NS.	NS.
WEEK 2.			
Group S (n=4)	14.92 ± 0.68	0.029 ± 0.004	0.095 ± 0.012
Group T(n=3)	6.86 ± 1.02	0.032 ± 0.002	0.038 ± 0.007
P(S vs T)	<0.005	NS.	<0.020
WEEK 3.			
Group S(n=5)	16.72 ± 0.74	0.034 ± 0.002	0.095 ± 0.007
Group T(n=4)	8.96 ± 1.94	0.026 ± 0.003	0.049 ± 0.006
Group S-T(n=5)	10.72 ± 1.60	0.028 ± 0.002	0.056 ± 0.004
Group T-S(n=4)	9.72 ± 1.40	0.030 ± 0.003	0.076 ± 0.013
P(S vs T)	<0.020	NS.	<0.005
P(S vs S-T)	<0.010	NS.	<0.005
P(S vs T-S)	<0.010	NS.	NS.
WEEK 4.			
Group S(n=5)	14.58 ± 1.18	0.033 ± 0.003	0.077 ± 0.007
Group T(n=5)	6.54 ± 0.56	0.024 ± 0.001	0.040 ± 0.006
Group S-T(n=4)	7.80 ± 0.76	0.028 ± 0.001	0.053 ± 0.006
Group T-S(n=4)	15.30 ± 0.80	0.036 ± 0.002	0.092 ± 0.011
P(S vs T)	<0.001	<0.0025	<0.005
P(S vs S-T)	<0.005	NS.	<0.050
P(S vs T-S)	NS.	NS.	NS.

Means ± SE.

†Cytochrome oxidase; µmoles cytochrome c oxidized/mg protein. min.
NADH-Cyt c. reductase; µmoles cytochrome c reduced/mg protein.min.

*Symbols are the same as in Table VIII.

amount of mitochondrial inner membrane has occurred, a rapid reduction in amount occurs, indicating that a very rapid turnover of these proteins occurs in the mitochondria in the brown adipose tissue of the cold-acclimated rat.

In skeletal muscle, the specific activity of cytochrome oxidase remains unchanged or decreases slightly in the saline-treated rats during the 4-week period of cold-acclimation and it is not significantly altered by the treatment with oxytetracycline except at week 4 (Figure 34 and Tables XV and XVI). Like cytochrome oxidase activity rotenone-sensitive NADH-cytochrome c reductase was not inhibited by oxytetracycline at week 2, 3 and 4. Also in muscle the rotenone-insensitive enzyme was not inhibited in the oxytetracycline-treated rats (Table XVI). The total amount of cytochrome oxidase in the muscle of the saline-treated rats (Table XV) during the period of cold-acclimation remains almost unchanged; only a small increase was observed which can be attributed to the increase in tissue size. On the other hand, in the oxytetracycline-treated rats the total amount of the enzyme in the muscle was significantly reduced at week 3 and 4, a decrease which can be attributed to a decrease in the concentration of enzyme at week 4 and to the slower rate of growth of the rats. In liver the specific activity of cytochrome oxidase of the saline-treated rats remains unchanged during the 4-weeks cold-acclimation period, but the treatment with the antibiotic causes a very marked inhibition at week 2, 3 and 4. It seems that the dose of oxytetracycline used in this experiment has an inhibitory

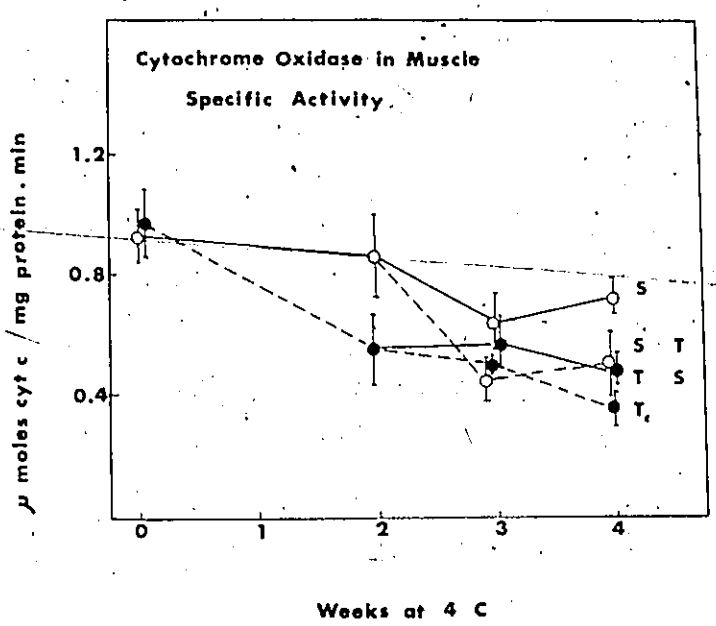


Figure 34. Effect of oxytetracycline administration during acclimation to cold on the specific activity of the cytochrome oxidase of skeletal muscle. For further details see the legend to Fig.32.

TABLE XV. Specific and total cytochrome oxidase activity in homogenate of muscle before and after 2, 3 and 4 weeks of cold-exposure with or without oxytetracycline treatment.

	Specific activity†	Total activity†
WEEK 0.		
Group S*(n=5)	0.93 ± 0.09	12616.92 ± 1126.50
Group T(n=5)	0.97 ± 0.12	12271.62 ± 1487.22
P(S vs T)	NS.	NS.
WEEK 2.		
Group S(n=4)	0.86 ± 0.14	15119.38 ± 2514.50
Group T(n=3)	0.55 ± 0.12	8919.46 ± 1067.66
P(S vs T)	NS.	NS.
WEEK 3.		
Group S(n=5)	0.64 ± 0.09	11828.16 ± 1763.84
Group T(n=4)	0.50 ± 0.03	6945.86 ± 598.00
Group S-T(n=5)	0.45 ± 0.07	7202.22 ± 1460.02
Group T-S(n=4)	0.57 ± 0.09	9901.04 ± 1670.48
P(S vs T)	NS.	<0.050
P(S vs S-T)	NS.	<0.050
P(S vs T-S)	NS.	NS.
WEEK 4.		
Group S(n=5)	0.72 ± 0.06	15762.00 ± 877.18
Group T(n=5)	0.35 ± 0.06	5295.28 ± 867.48
Group S-T(n=4)	0.50 ± 0.11	7600.28 ± 1191.96
Group T-S(n=4)	0.48 ± 0.05	9643.18 ± 484.24
P(S vs T)	<0.005	<0.001
P(S vs S-T)	NS.	<0.001
P(S vs T-S)	<0.025	<0.001

Means ± SE.

†Specific activity; µmoles cytochrome c oxidized/mg protein.min.
Total; µmoles cytochrome c oxidized per min by total organ.
For the calculations it is assumed that muscle represent 43.3% of body weight (Heroux, 1958)

*Symbols are the same as in Table VIII

TABLE XVI. Specific activity of cytochrome oxidase, NADH-cytochrome c reductase (Rotenone sensitive and insensitive) in muscle mitochondria before and after 2,3 and 4 weeks of cold-exposure with or without oxytetracycline treatment.

	Cytochrome Oxidase†	NADH-cyt c Red. Rot.insensitive†	NADH-cyt c Red. Rot. sensitive.†
WEEK 0.			
Group S*(n=5)	7.84 ± 0.50	0.020 ± 0.005	0.132 ± 0.026
Group T(n=5)	8.68 ± 0.82	0.020 ± 0.004	0.133 ± 0.025
P(S vs T)	NS.	NS.	NS.
WEEK 2.			
Group S(n=4)	11.76 ± 0.99	0.034 ± 0.003	0.173 ± 0.029
Group T(n=3)	8.90 ± 0.42	0.026 ± 0.001	0.112 ± 0.015
P(S vs T)	<0.050	NS.	NS.
WEEK 3.			
Group S(n=5)	9.50 ± 1.57	0.025 ± 0.003	0.116 ± 0.011
Group T(n=4)	7.64 ± 0.86	0.023 ± 0.003	0.112 ± 0.019
Group S-T(n=5)	8.50 ± 1.03	0.026 ± 0.008	0.104 ± 0.019
Group T-S(n=4)	7.18 ± 0.92	0.026 ± 0.006	0.096 ± 0.020
P(S vs T)	NS.	NS.	NS.
P(S vs S-T)	NS.	NS.	NS.
P(S vs T-S)	NS.	NS.	NS.
WEEK 4.			
Group S(n=5)	9.72 ± 1.48	0.029 ± 0.006	0.079 ± 0.016
Group T(n=5)	6.18 ± 0.53	0.020 ± 0.004	0.079 ± 0.009
Group S-T(n=4)	7.36 ± 1.07	0.031 ± 0.006	0.080 ± 0.008
Group T-S(n=4)	10.54 ± 0.81	0.034 ± 0.007	0.105 ± 0.004
P(S vs T)	NS.	NS.	NS.
P(S vs S-T)	NS.	NS.	NS.
P(S vs T-S)	NS.	NS.	NS.

Means ± SE

†Cytochrome oxidase; µmoles cytochrome c oxidized/mg protein.min.
NADH-Cyt c. reductase; µmoles cytochrome c reduced/mg protein.min.

*Symbols are the same as in Table VIII

TABLE XVII. Specific and total cytochrome oxidase activity in homogenate of liver before and after 2, 3 and 4 weeks of cold exposure with or without oxytetracycline treatment.

	Specific activity†	Total activity†
WEEK 0.		
Group S*(n=5)	3.65 ± 0.10	4269.30 ± 190.42
Group T(n=5)	2.94 ± 0.22	3872.28 ± 322.22
P(S vs T)	<0.020	NS.
WEEK 2.		
Group S(n=4)	3.76 ± 0.25	7990.82 ± 388.02
Group T(n=3)	1.56 ± 0.10	2761.46 ± 273.04
P(S vs T)	<0.005	<0.005
WEEK 3.		
Group S(n=5)	3.38 ± 0.20	6479.02 ± 344.54
Group T(n=4)	1.39 ± 0.17	2129.04 ± 337.96
Group S-T(n=5)	1.71 ± 0.14	2770.50 ± 110.54
Group T-S(n=4)	2.26 ± 0.12	4605.90 ± 315.16
P(S vs T)	<0.001	<0.001
P(S vs S-T)	<0.005	<0.001
P(S vs T-S)	<0.010	<0.005
WEEK 4.		
Group S(n=5)	2.93 ± 0.11	7118.24 ± 505.48
Group T(n=5)	0.94 ± 0.08	1667.24 ± 206.48
Group S-T(n=4)	1.21 ± 0.10	2432.92 ± 273.76
Group T-S(n=4)	3.21 ± 0.10	7930.74 ± 343.02
P(S vs T)	<0.001	<0.001
P(S vs S-T)	<0.001	<0.001
P(S vs T-S)	NS.	NS.

Means ± SE.

†Specific activity; µmoles cytochrome oxidized/mg protein.min.
Total: µmoles cytochrome c oxidized per min by total organ.

*Symbols are the same as in Table VIII.

effect on the protein synthesis of liver mitochondria. It should be noted that when partially cold-acclimated rats (Group S, week 2) were treated with the antibiotic for 1 or 2 weeks the cytochrome oxidase activity is also reduced (Group S-T, weeks 3 and 4) (see Table xvii), and that when the treatment was suspended (Group T, week 2) the cytochrome oxidase activity is restored to normal values (Group T-S, weeks 3 and 4). The total amount of cytochrome oxidase in liver was increased almost 2 times during the first two weeks of cold-exposure in the saline-treated rats (see Table XVII) and remains unchanged at weeks 3 and 4, but this increase is due only to the increase in the total amount of tissue because the concentration of the enzyme remains the same. In the oxytetracycline-treated rats the total amount of cytochrome oxidase in liver was decreased at week 2 and more markedly at weeks 3 and 4; these decreases can be attributed to the decrease in enzyme concentration and also to the slight decrease in the organ size at week 3 and 4.

Thus, the inhibition of mitochondrial protein synthesis by oxytetracycline treatment appears to be more specific for brown adipose tissue, in which rapid proliferation of the mitochondria occurs during cold-acclimation. However it can be expected that the treatment would also inhibit mitochondrial protein synthesis in all the tissues and this inhibition may not be detected by measuring cytochrome oxidase and NADH-cytochrome c reductase (rotenone-sensitive). In fact the failure to detect inhibition of cytochrome oxidase in muscle can be due to the

lack of change in the amount of this enzyme during cold-acclimation. The constancy of this enzyme in muscle during cold-acclimation was previously demonstrated in Section A. The slight inhibition observed at week 2 could be due to the occurrence at this time of major changes in the mitochondria which could affect in some way, not the amount of the enzyme but its stability or activation or binding to the membrane; when the changes have stabilized the enzyme may be no longer susceptible to inhibition, which can explain the lack of inhibition by the antibiotic at week 3 and 4.

That mitochondrial protein synthesis is related to the development or/and the maintenance of nonshivering thermogenesis can be found in the fact that its inhibition by oxytetracycline also inhibits the development and maintenance of the enhanced calorigenic response to noradrenaline. By comparing directly Figure 33 with Figure 32 it can be observed that when mitochondrial protein synthesis is inhibited during acclimation to cold (weeks 0-4) the development and maintenance of the enhanced response to noradrenaline is also inhibited in the same group of rats (compare Group T, week 2, 3 and 4, with group S). These results are in close accord with those of Himms-Hagen (1971).

On the other hand, when the antibiotic was withdrawn after two weeks of treatment during cold-acclimation the mitochondrial protein synthesis was resumed and the metabolic response to noradrenaline quickly enhanced to normal values. If the increased mitochondrial protein synthesis is first allowed to occur (Group S, week 2), subsequent treatment with the antibiotic causes a rapid disappearance of the extra cytochrome oxidase

activity and also the elevated calorigenic response to noradrenaline disappears rapidly (T-S, week 3 and 4).

It can be suggested then that as a part of, or related to the mechanism of nonshivering thermogenesis, an acceleration in the synthesis of mitochondrial protein takes place during cold-acclimation and is associated with the development and the maintenance of the enhanced response to noradrenaline induced by cold-acclimation. These results, together with those of Bukowiecki and Himms-Hagen (1971) and Himms-Hagen (1971), suggest that accelerated synthesis of mitochondrial protein might occur in association with the development and the maintenance of the cold-acclimated state.

Section C. MORPHOLOGY AND BIOCHEMICAL PROPERTIES OF MUSCLE MITOCHONDRIA IN RATS IN WHICH THE ADAPTATION FOR NON-SHIVERING THERMOGENESIS IS PREVENTED BY OXYTETRACYCLINE.

a) Purpose of the experiment: To find out whether the increase in number and decrease in size of muscle mitochondria in cold-acclimated rats occurs in association with the development of the capacity for nonshivering thermogenesis (enhanced metabolic response to noradrenaline) or occurs as a consequence of shivering. The development of the capacity for nonshivering thermogenesis will be inhibited with oxytetracycline, as described in Section B, and the effect of this treatment on the usual increase in number and decrease in size of muscle mitochondria observed.

b) Description of the experiment: This experiment is essentially similar to the experiment described in Section B of this Chapter, with the exception that the rats underwent cold-acclimation for a period of only 2 weeks. A corresponding group of warm-acclimated rats was also studied. Instead of following the calorigenic response to noradrenaline as an index of cold-acclimation (as described in Section B) the number and size of muscle mitochondria were measured.

32 Male rats were divided in 2 groups of 16 rats each, warm-acclimated; 136.75 ± 2.40 g, and cold-acclimated; 140.00 ± 2.72 g. Both groups were subdivided in 2 subgroups; warm, Group S (8 rats); warm, Group T (8 rats); cold, Group S (8 rats) and cold, Group T (8 rats). The rats remained at their respective temperature of acclimation for 2 weeks receiving saline injections (Groups S) or oxytetracycline injections (Groups T). The daily total dose of oxytetracycline was 200 mg/kg (given in two doses).

From each group, 3 rats were used for the histological and electron microscopic studies and the rest of the rats were used for the isolation of mitochondria from about 10 g of mixed muscle. Because it was not feasible to use a sufficient number of rats in this experiment in order to isolate mitochondria from the red and white portions of the semitendinosus, it was decided to use mixed muscle instead of studying red and white muscle separately. About 10 g of muscle from the legs and back of each rat were taken for the isolation of mitochondria.

c) Results and discussion: The histochemical demonstration of succinic dehydrogenase showed that the gross appearance of the fibers of the semitendinosus muscle were not affected significantly by the treatment with oxytetracycline in the warm-acclimated rats. On the other hand all the changes in the gross appearance of the fibers (reduction in size of the fiber, increase proportion of red fibers, transformation of white fibers in intermediate type) which occurred in cold-acclimated rats (Group S) were prevented by the treatment with oxytetracycline (Group T).

The electron microscopic studies revealed a marked effect on the ultrastructure of the mitochondria of the partially cold-acclimated rats treated with the antibiotic. Figures 35, 36, 37 and 38 shown representative electronmicrographs of red and white semitendinosus muscle from saline-treated and oxytetracycline-treated warm-acclimated and cold-acclimated rats.

No significant effect of the antibiotic treatment on the ultrastructure of red or white muscle of warm-acclimated rats was observed (Figures 35 and 36; compare C with A and D with B in each Figure). Only a few mitochondria appear to be affected. In general the mitochondria conserve their organization and the cristae are normal.

The normal changes due to the acclimation to cold were less marked in the saline-treated rats than had been observed previously (Section A). This is probably because the period of acclimation was rather short (only two weeks). Nevertheless, the antibiotic appeared to prevent the usual changes

Figure 35. Electron micrographs of semitendinosus muscle from:

(A) Warm-acclimated rat,saline-treated rat; red fiber

(B) Warm-acclimated rat,saline-treated rat; white fib

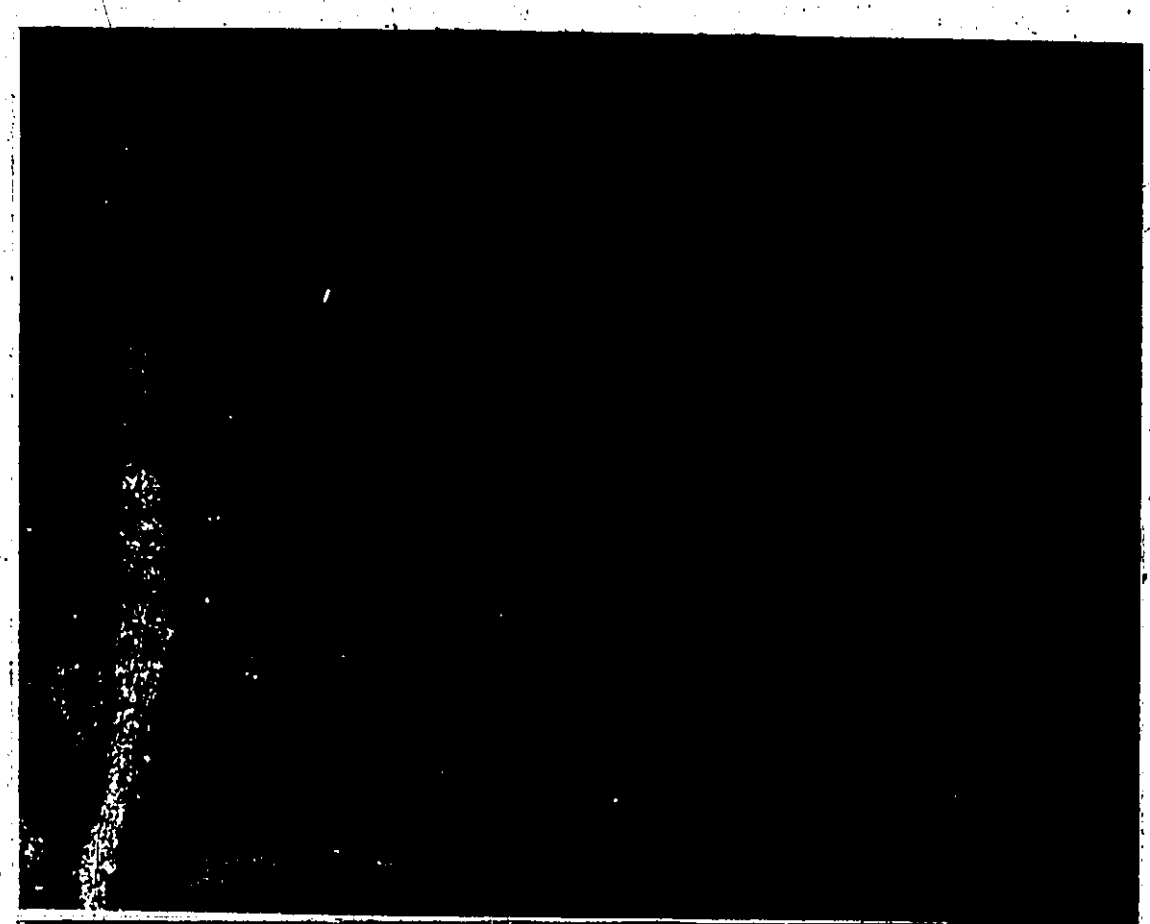
(C) Warm-acclimated rat,oxytetracycline-treated rat; red fiber,

(D) Warm-acclimated rat,oxytetracycline-treated rat; white fiber,

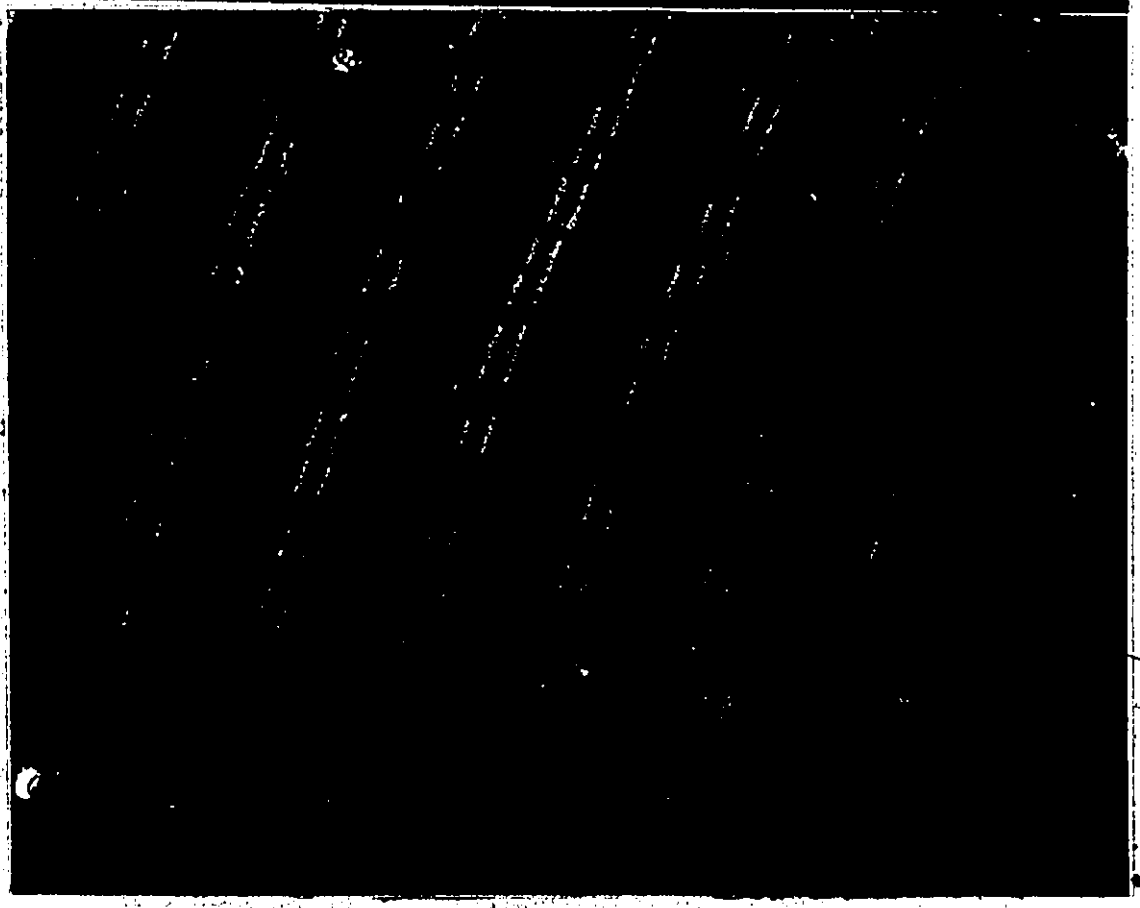
No significant effect of the antibiotic treatment on t ultrastructure of red or white muscles of warm-acclimated rats can be observed. Only a few mitochondr appear to be affected. In general the mitochondria conserve their organization and the cristae are normal

Taken at 7000 X magnification with the electron microscope: magnification in printing X 2.

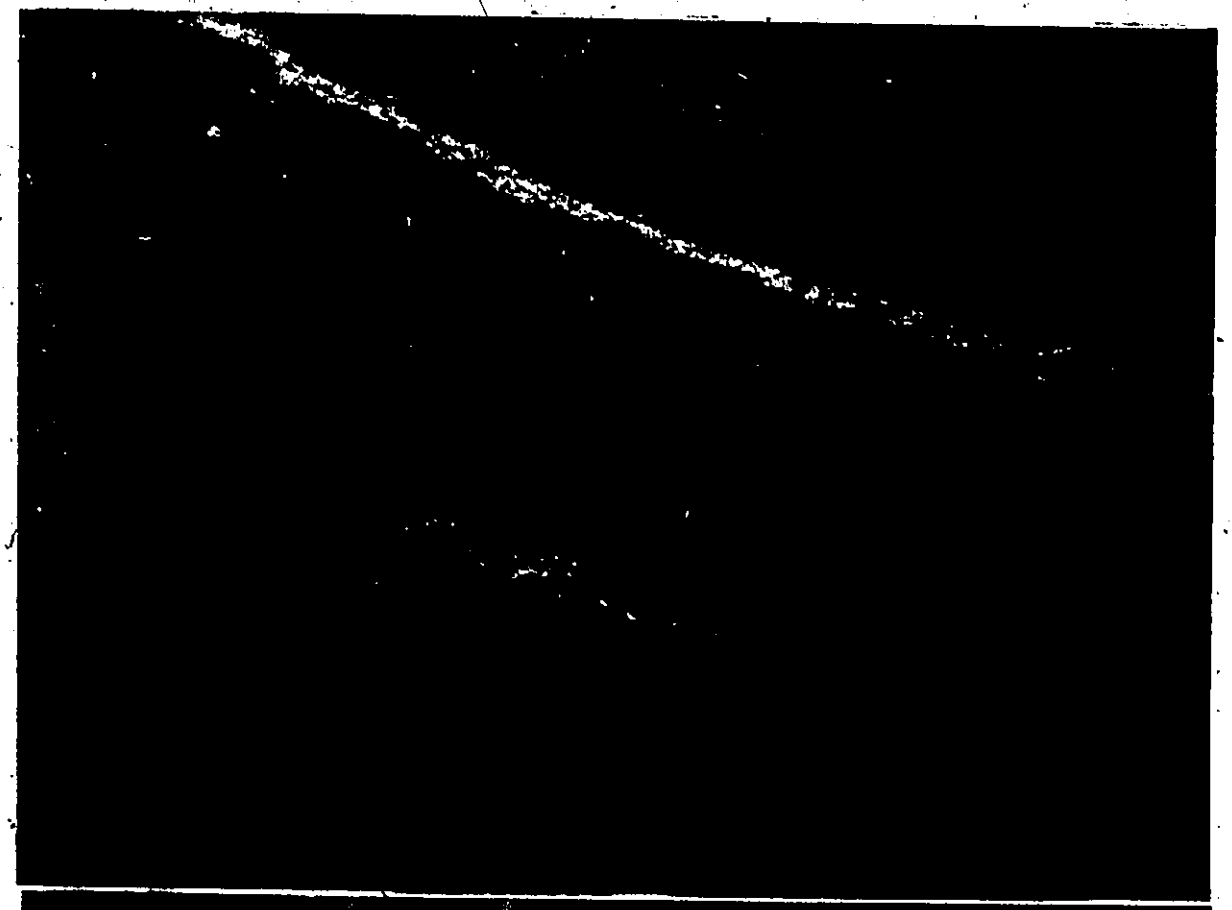
(A)



(B)



(C)



(D)

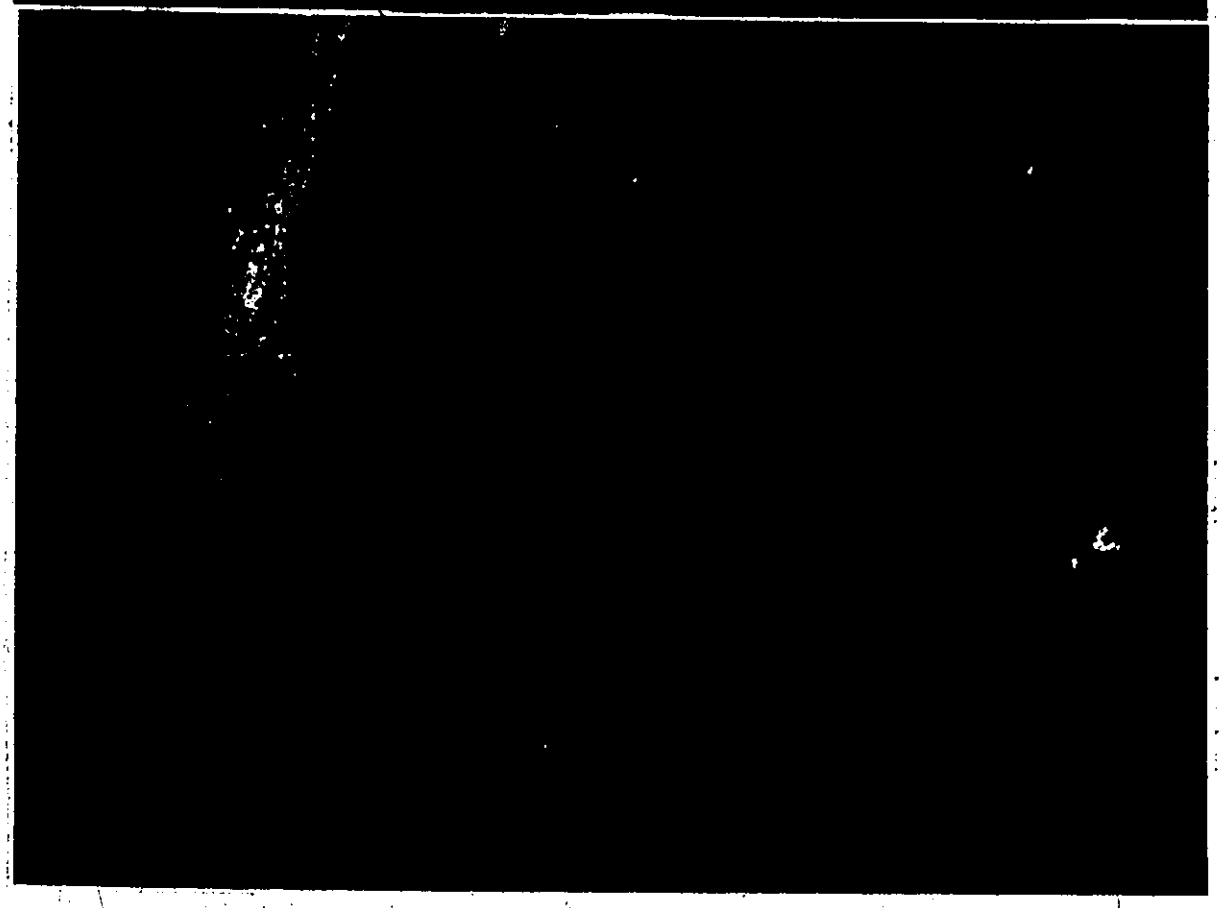
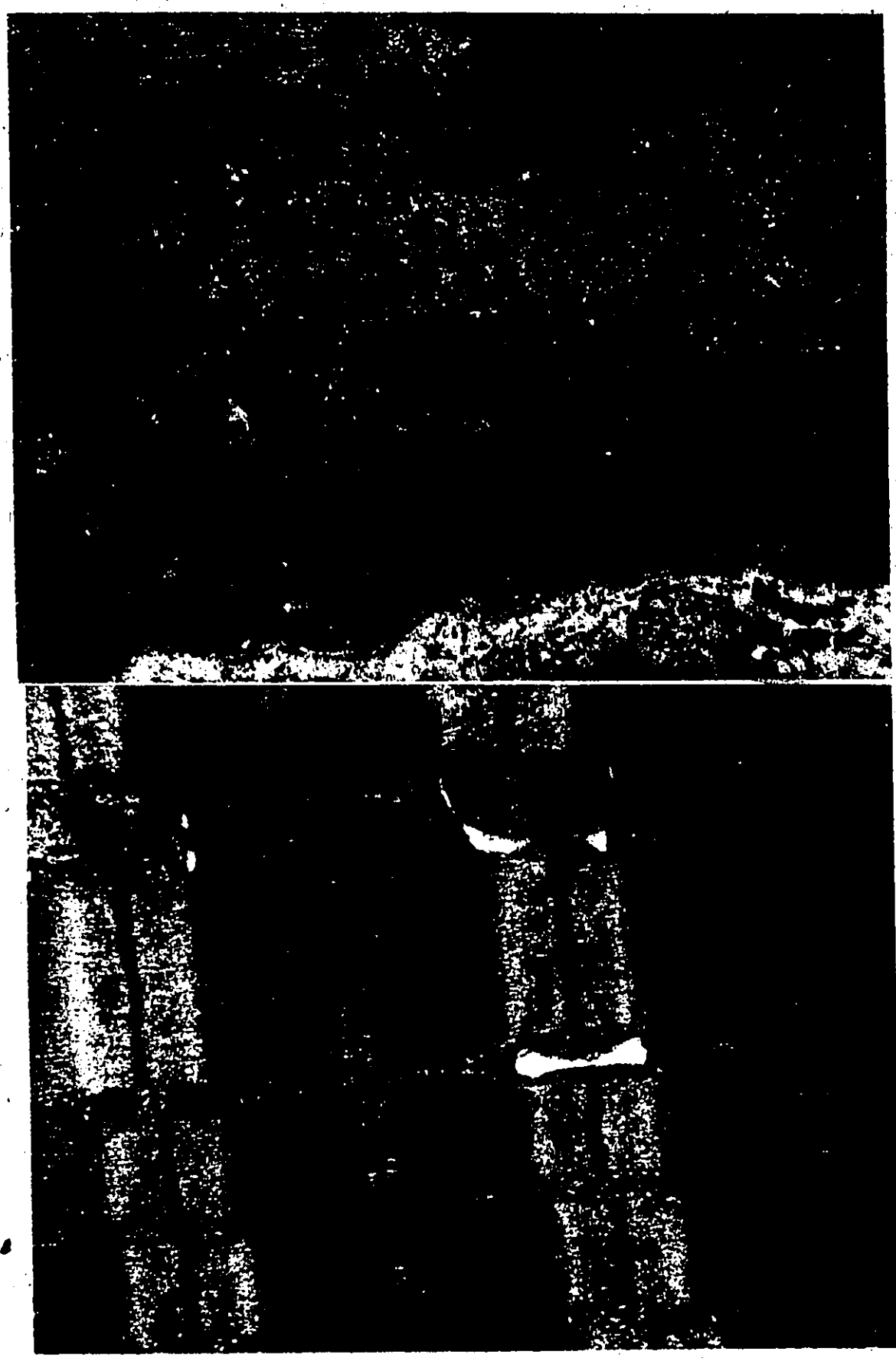


Figure 36. Electron micrographs of semitendinosus muscle from:

(A) Warm-acclimated,saline-treated rat; red fiber,
(B) Warm-acclimated,saline-treated rat; white fiber,
(C) Warm-acclimated,oxytetracycline-treated rat; red fiber,
(D) Warm-acclimated,oxytetracycline-treated rat; white fiber.

Taken at 20000 X magnification with the electron microscope: magnification in printing X 2.



(A)

(B)

(C)

(D)

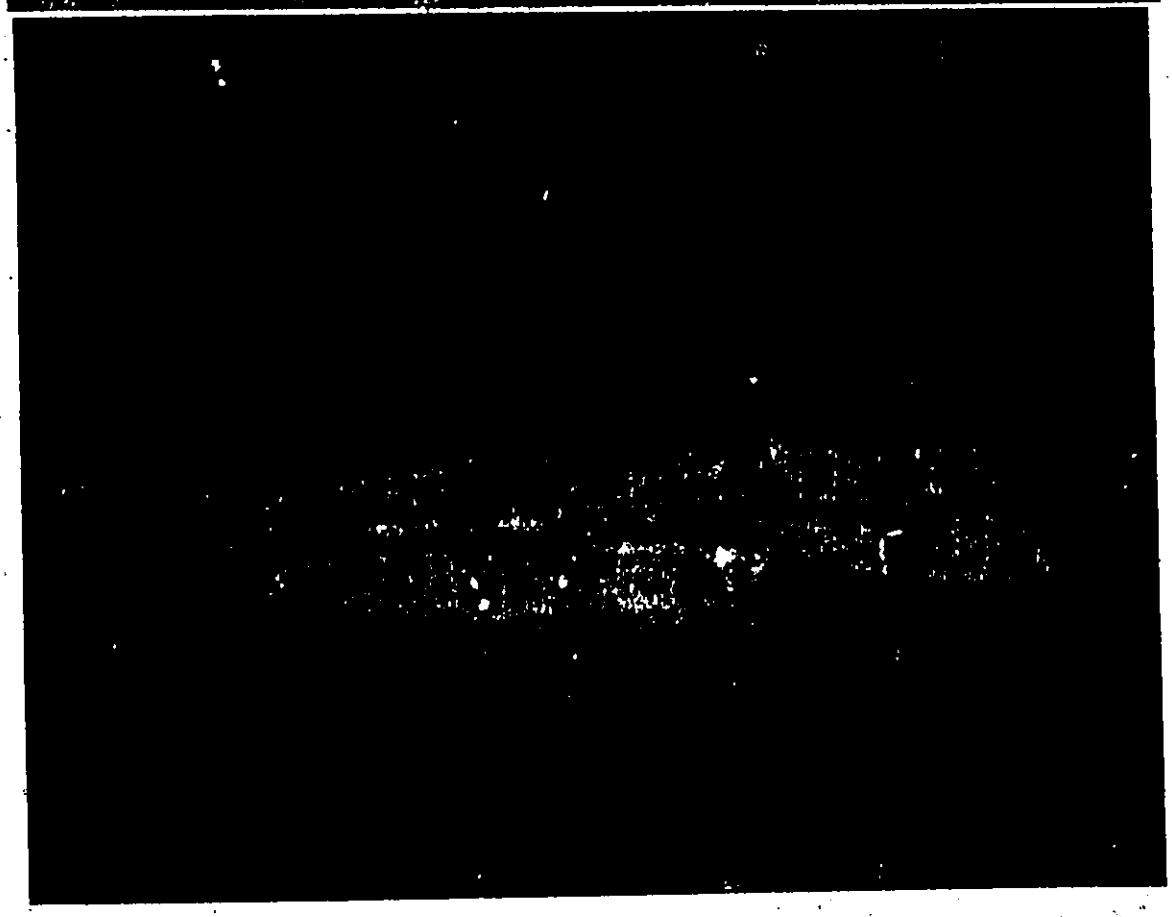


Figure 37. Electron micrographs of semitendinosus muscle from:

(A) Cold-acclimated,saline-treated rat,red fiber,

(B) Cold-acclimated,saline-treated rat,white fiber,

The normal changes due to the acclimation to cold are less marked in these saline-treated rats than had been observed previously.,i.e.,large mitochondria are still present. This is probably because the period of acclimation was rather short (two weeks).

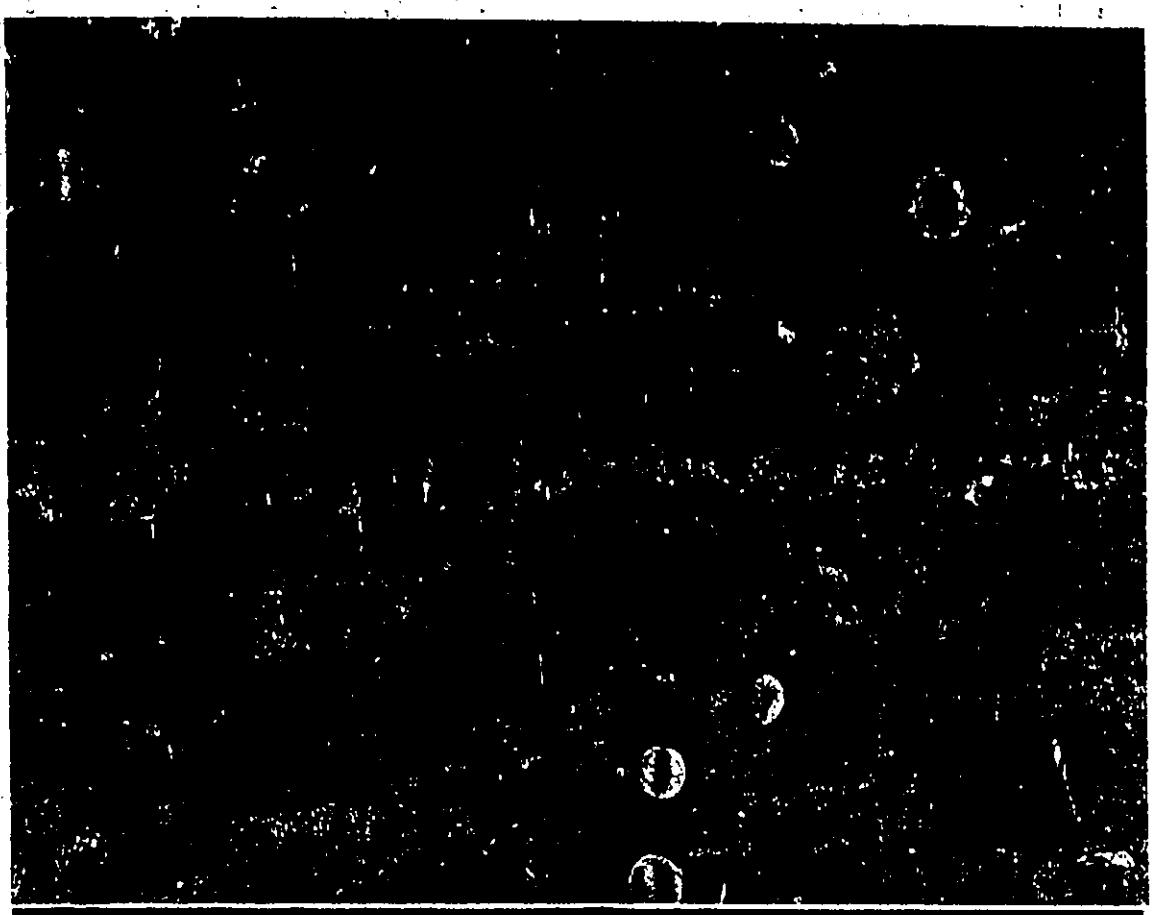
(C) Cold-acclimated,oxytetracycline-treated rat, red fiber,

(D) Cold-acclimated,oxytetracycline-treated rat, white fiber,

The antibiotic appeared to prevent the usual changes observed in cold-acclimated muscles. No increase in number of mitochondria is apparent in either red or white muscle of the oxytetracycline-treated rat. The mitochondria which remain,especially the small ones, seem to be drastically affected by the antibiotic.

Taken at 7000 X magnification with the electron microscope: magnification in printing X 2.

(A)



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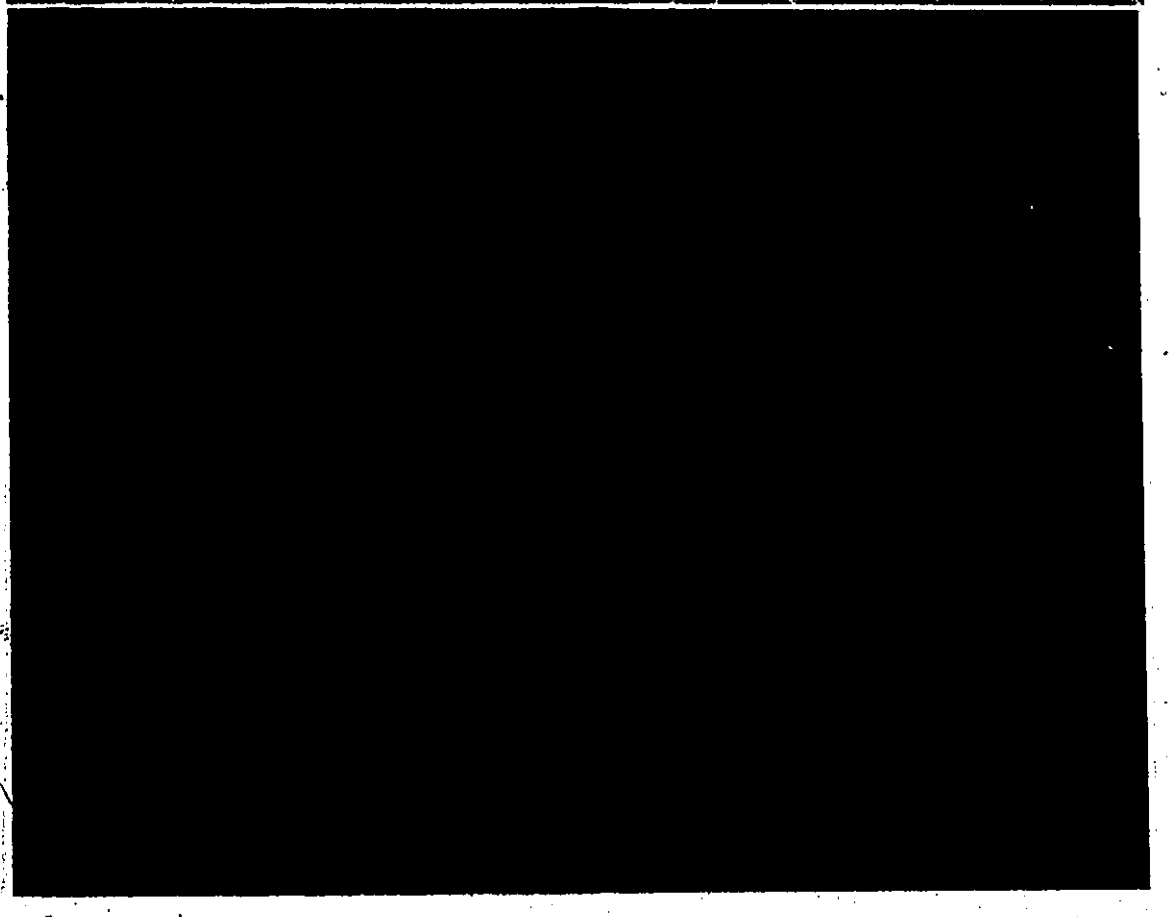
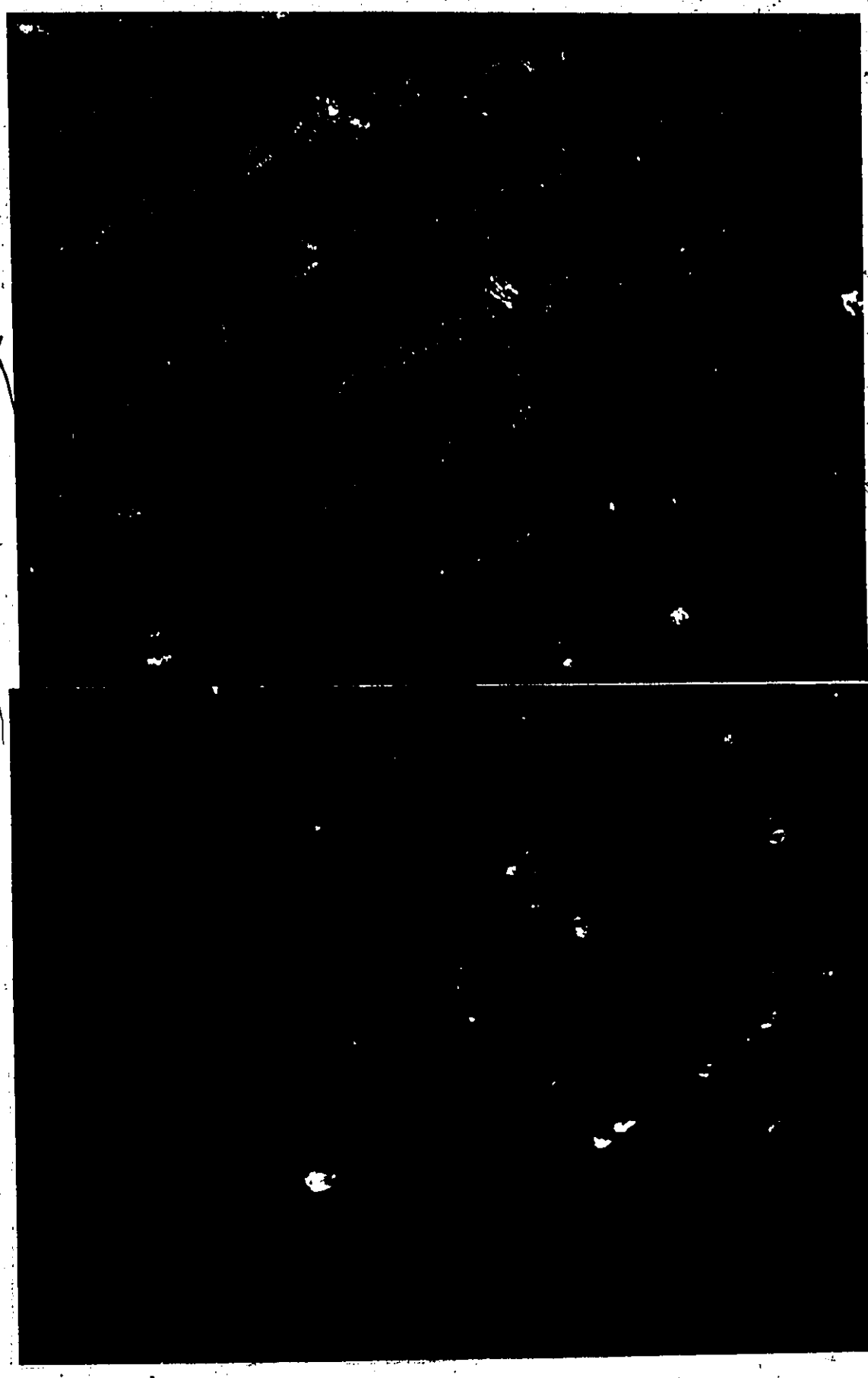




(C)

(D)

Figure 38. Electron micrographs of semitendinosus muscle from:

(A) Cold-acclimated,saline-treated rat,red fiber

(B) Cold-acclimated,saline-treated rat,white fiber,

The normal changes due to the acclimation to cold are less marked in these saline-treated rats than had been observed previously probably because the period of acclimation was rather short (only two weeks).

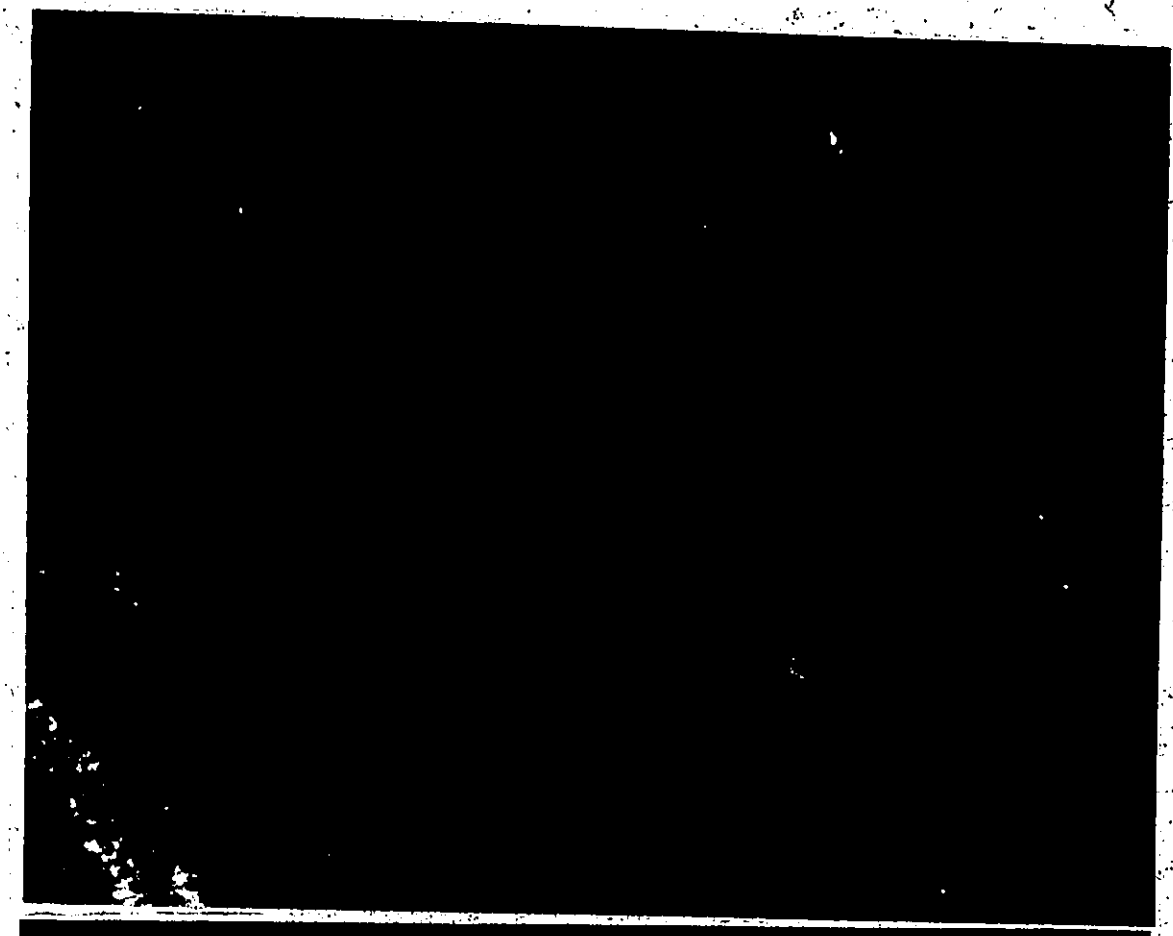
(C) Cold-acclimated,oxytetracycline-treated rat, red fiber.

(D) Cold-acclimated,oxytetracycline-treated rat, white fiber,

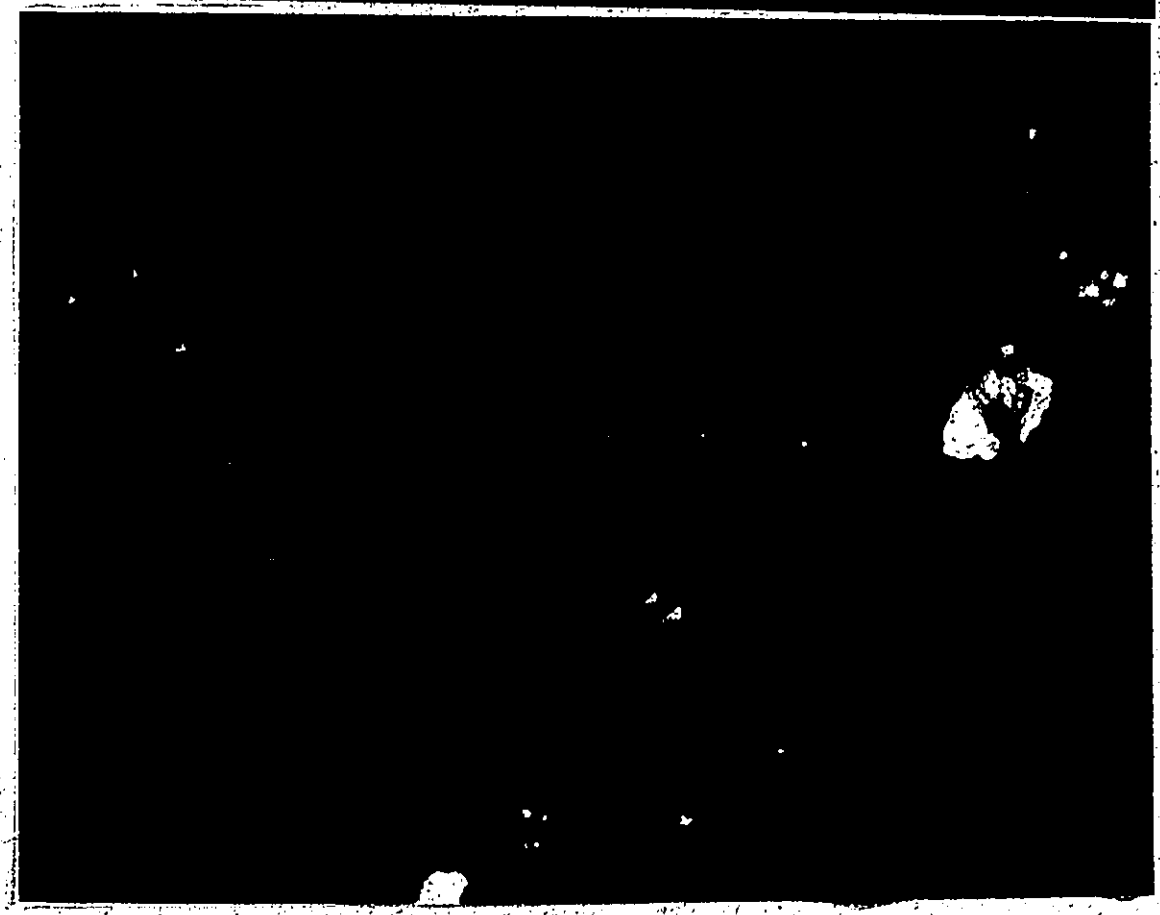
The antibiotic appeared to prevent the usual changes observed in cold-acclimated muscles. No increase in number of mitochondria is observed. The mitochondria which remain seem to be drastically affected by the antibiotic. Note the distorted inner mitochondrial membrane and the relatively sparse cristae. Especially interesting is the occurence of circular inner membranes (D),where the structures,have apparently broken loose from much of the outer membrane.

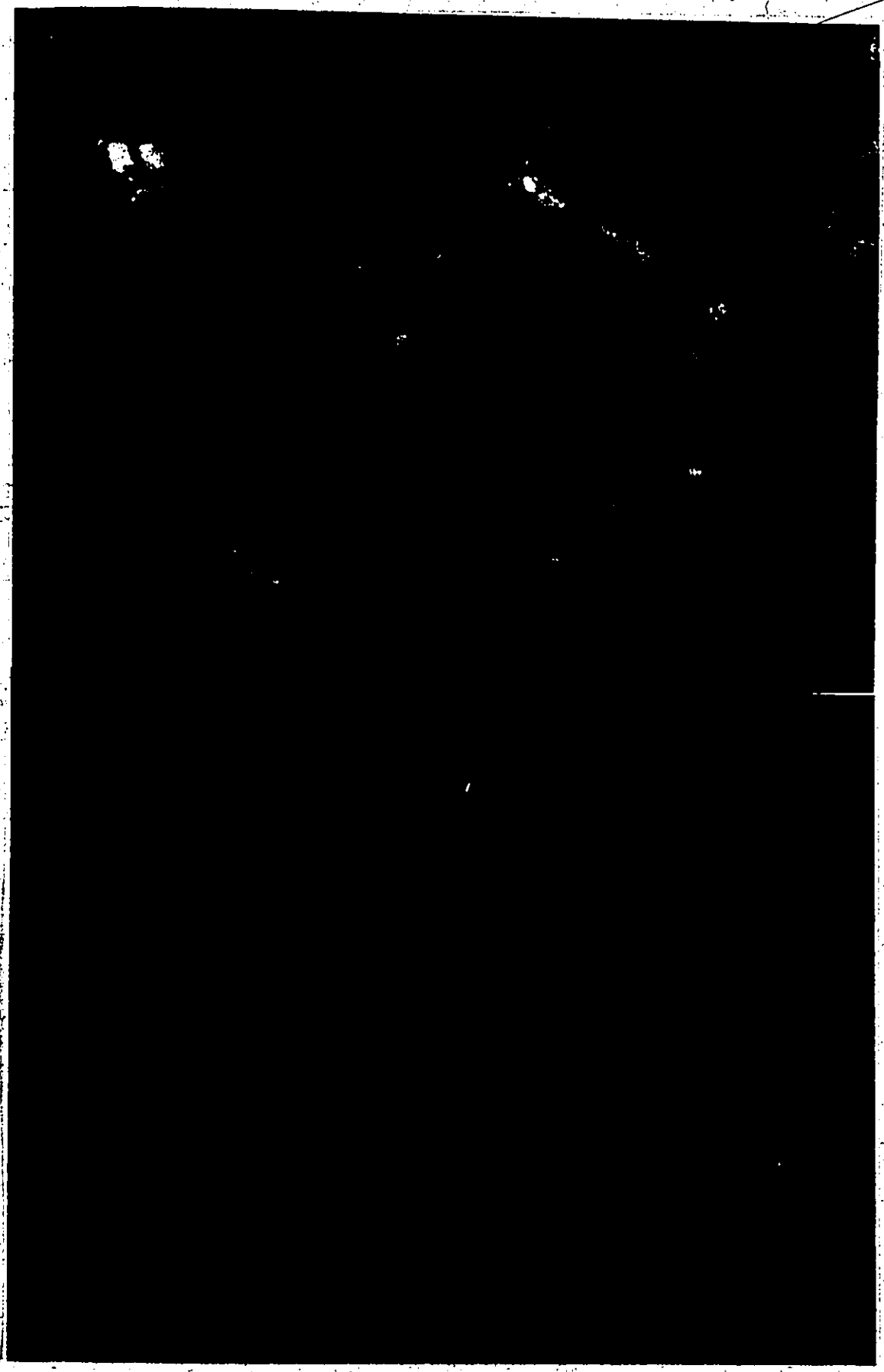
Taken at 20000 X magnification with the electron microscope: magnification in printing X 2.

(A)



(B)





(C)

(D)

observed in cold-acclimated muscles (Figures 37 and 38; compare C with A and D with B in each Figure). No increase in number of mitochondria is apparent in either red or white muscle of the oxytetracycline-treated rats. The mitochondria which remain, especially the small ones, seem to be drastically affected by the antibiotic. Note the distorted inner mitochondrial membrane and the relatively sparse cristae. Especially interesting is the occurrence of circular inner membranes, where the structures have apparently broken loose from much of the outer membrane. These alterations are characteristic of antibiotic-treated cells and are not seen in control cells. Similar results have been found with the mitochondria from yeast treated with chloramphenicol (Clark and Linnane, 1967) and with the mitochondria of HeLa cells incubated in chloramphenicol or ethidium bromide (Lenk and Penman, 1971).

Counting the mitochondria isolated from the mixed muscle of saline-treated and oxytetracycline-treated cold-acclimated rats showed that the usual increase in mitochondrial number induced by acclimation to cold was prevented by the treatment with oxytetracycline (Figure 39). There was no effect of the antibiotic treatment on mitochondrial number in muscle of warm-acclimated rats (Figure 39).

Further information about the isolated mitochondria is given in Table XVIII. The usual decrease in size of the mitochondria (protein content per mitochondrion, cytochrome oxidase activity per mitochondrion) was prevented by the treatment of the cold-acclimated rats with oxytetracycline. The size of

the mitochondria in the muscle of the warm-acclimated rats was not altered by the antibiotic-treatment.

The specific activity of cytochrome oxidase in the isolated mitochondria was not altered by treatment with oxytetracycline in either cold-acclimated or warm-acclimated rats. A slight and similar reduction in total cytochrome oxidase per gram of muscle occurred in muscle of both warm-acclimated and cold-acclimated rats as a consequence of the treatment with oxytetracycline.

Three principal conclusions can be drawn from these results:

1. The increase in number and decrease in size of muscle mitochondria in the cold-acclimated rat is associated with the development of the adaptation for nonshivering thermogenesis (enhanced metabolic response to noradrenaline) because inhibition of the development of the adaptation is accompanied by inhibition of the appearance of the increase in number of muscle mitochondria.
2. The increase in number and decrease in size of muscle mitochondria does not occur as a consequence of shivering. The antibiotic-treated rats have a high metabolic rate in the cold and are presumably shivering since they cannot use nonshivering thermogenesis. Nevertheless, no change in muscle mitochondria is apparent in these shivering rats.
3. Mitochondrial protein synthesis is necessary for the

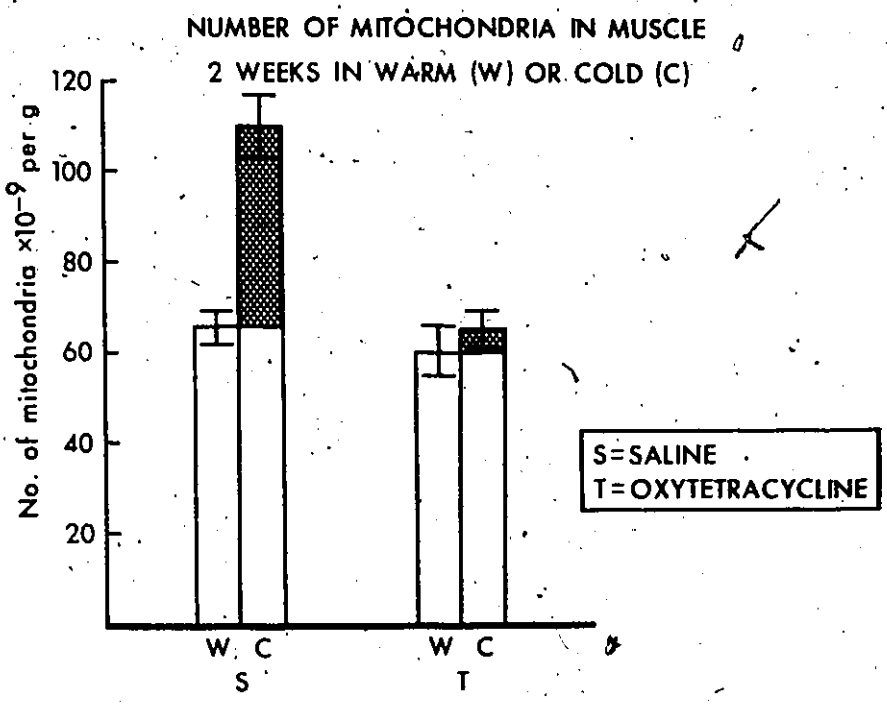


Figure 39. Number of mitochondria in mixed muscle of rats receiving daily injections of saline (S) or oxytetracycline (T) during two weeks in the warm (W) or in the cold (C)

TABLE XVIII. Number of mitochondria and biochemical parameters of isolated mitochondria from mixed muscle of saline-treated and oxytetracycline-treated rats from 2 weeks warm-acclimated and cold-acclimated rats.

	Saline-treated	OTC-treated*	P (Saline vs OTC)
Recovery:mg mitoch protein/g muscle			
WARM-ACCLIMATED	3.10 ± 0.22	2.22 ± 0.41	NS.
COLD-ACCLIMATED	2.91 ± 0.22	2.03 ± 0.24	<0.050
P(warm vs cold)	NS.	NS.	
Recovery:cytochrome oxidase,%			
WARM-ACCLIMATED	34.81 ± 3.01	29.16 ± 3.70	NS.
COLD-ACCLIMATED	30.27 ± 2.51	27.16 ± 1.20	NS.
P(warm vs cold)	NS.	NS.	
Total mitochondrial protein/g muscle			
WARM-ACCLIMATED	9.144 ± 0.973	7.666 ± 0.949	NS.
COLD-ACCLIMATED	9.650 ± 1.063	7.407 ± 0.683	NS.
P(warm vs cold)	NS.	NS.	
Protein:picograms/ mitochondrion			
WARM-ACCLIMATED	0.1387 ± 0.0099	0.1238 ± 0.0097	NS.
COLD-ACCLIMATED	0.0879 ± 0.0083	0.1136 ± 0.0051	<0.050
P(warm vs cold)	<0.001	NS.	

...continued

TABLE XVIII. (continuation)

	Saline-treated	OTC-treated*	P (Saline vs OTC)
No mitochondria/g muscle (10^{-9})			
WARM-ACCLIMATED	65.619 ± 3.578	60.682 ± 5.272	NS.
COLD-ACCLIMATED	110.065 ± 6.861	64.936 ± 4.215	<0.001
P(warm vs cold)	<0.001	NS.	
Specific activity cytochrome oxidase µatoms O_2/mg prot.min.			
WARM-ACCLIMATED	17.794 ± 2.383	16.816 ± 1.695	NS.
COLD-ACCLIMATED	18.612 2.985	17.242 ± 2.056	NS.
P(warm vs cold)	NS.	NS.	
No mitochondria/mg mitochondrial protein (10^{-9})			
WARM-ACCLIMATED	7.359 ± 0.528	8.133 ± 0.577	NS.
COLD-ACCLIMATED	11.855 ± 1.340	8.885 ± 0.433	<0.100 (NS)
P(warm vs cold)	<0.020	NS.	
cytochrome oxidase µatoms O_2/ mitochondrion (10^9)			
WARM-ACCLIMATED	2.416 ± 0.240	2.053 ± 0.079	NS.
COLD-ACCLIMATED	1.547 ± 0.107	1.918 ± 0.125	<0.100 (NS)
P(warm vs cold)	<0.020	NS.	
cytochrome oxidase µatoms O_2/g muscle.min.			
WARM-ACCLIMATED	157.12 ± 13.46	123.92 ± 9.88	<0.100 (NS)
COLD-ACCLIMATED	172.66 ± 12.11	123.16 ± 9.04	<0.020
P(warm vs cold)	NS.	NS.	

Means ± SE. n = 5. *OTC-treated:oxytetracycline treated.

increase in number and decrease in size of muscle mitochondria to occur during cold-acclimation.

Section D. MORPHOLOGY AND BIOCHEMICAL PROPERTIES OF MUSCLE MITOCHONDRIA IN COLD-ACCLIMATED RATS IN WHICH THE ADAPTATION FOR NONSHIVERING THERMOGENESIS HAS BEEN REVERSED BY OXYTETRACYCLINE.

a) Purpose of the experiment: To find out whether the increase in number and decrease in size of muscle mitochondria in cold-acclimated rats disappears when the adaptation for nonshivering thermogenesis (enhanced metabolic response to noradrenaline) which has occurred in these animals is reversed by treatment with oxytetracycline, as described in Section B.

b) Description of the experiment: The experiment described here was essentially the same as that described in Section B. The studies on histochemistry, ultrastructure and isolation of mitochondria were carried out in rats subjected to the same conditions of Group S (week 4) and Group S-T (week 4).

10 Male rats were placed in the cold room when their body weight was 127.50 ± 3.48 g for a period of 2 weeks. At the end of this time they were separated into two groups; Group S (5 rats) and Group S-T (5 rats). The rats from the Group S received daily injections of saline solution and the rats of the Group S-T received daily injections of oxytetracycline (200 mg/kg) for two further weeks as described previously. At the end of this time 2 rats from each group were

used for the histological and electron microscopic observations and the remaining 3 rats in each group were used for the isolation of mitochondria from mixed muscle (about 10 g of muscle were collected from each rat). The final body weights were, Group S (week 4) 214.80 ± 11.63 g and Group S-T (week 4) 215.80 ± 3.88 g.

c) Results and Discussion: The histological studies showed that in the saline-treated rats (Group S, week 4) which had been living in the cold for 4 weeks all the previously described changes took place with respect to fiber size and distribution. However the gross characteristics of the fibers of the oxytetracycline-treated rats (Group S-T, week 4) were different. The fibers appeared smaller in size but a reduction in the number of red fibers was observed. The electron microscopic observations (Figure 40) showed that the treatment with oxytetracycline affected both types of fibers, red and white. The mitochondria showed a generalized disorganization of the cristae, with some circular inner membrane; the outer membrane seemed not to be affected. Also a reduction in the number of mitochondria was apparent with a general predominance of the big mitochondria in the oxytetracycline-treated rats, in comparison with those of Group S which showed the normal increase in number and reduction in the size of the mitochondria during the 4-weeks period of cold-acclimation.

The counting of mitochondria (Figure 41 and Table XIX) corroborates the electron microscopic observations. The number of mitochondria was reduced in these rats which were

Figure 40. Electron micrographs of semitendinosus muscle from:

(A) Cold-acclimated,saline-treated rat (4 weeks); red fiber, (S)*,

(B) Cold-acclimated,saline-treated rat (4 weeks); white fiber, (S)*,

These rats show the normal increase in number and reduction in the size of the muscle mitochondria during the 4-week period of cold-acclimation.

(C) Cold-acclimated,oxytetracycline-treated rat, red fiber, (S-T)*,

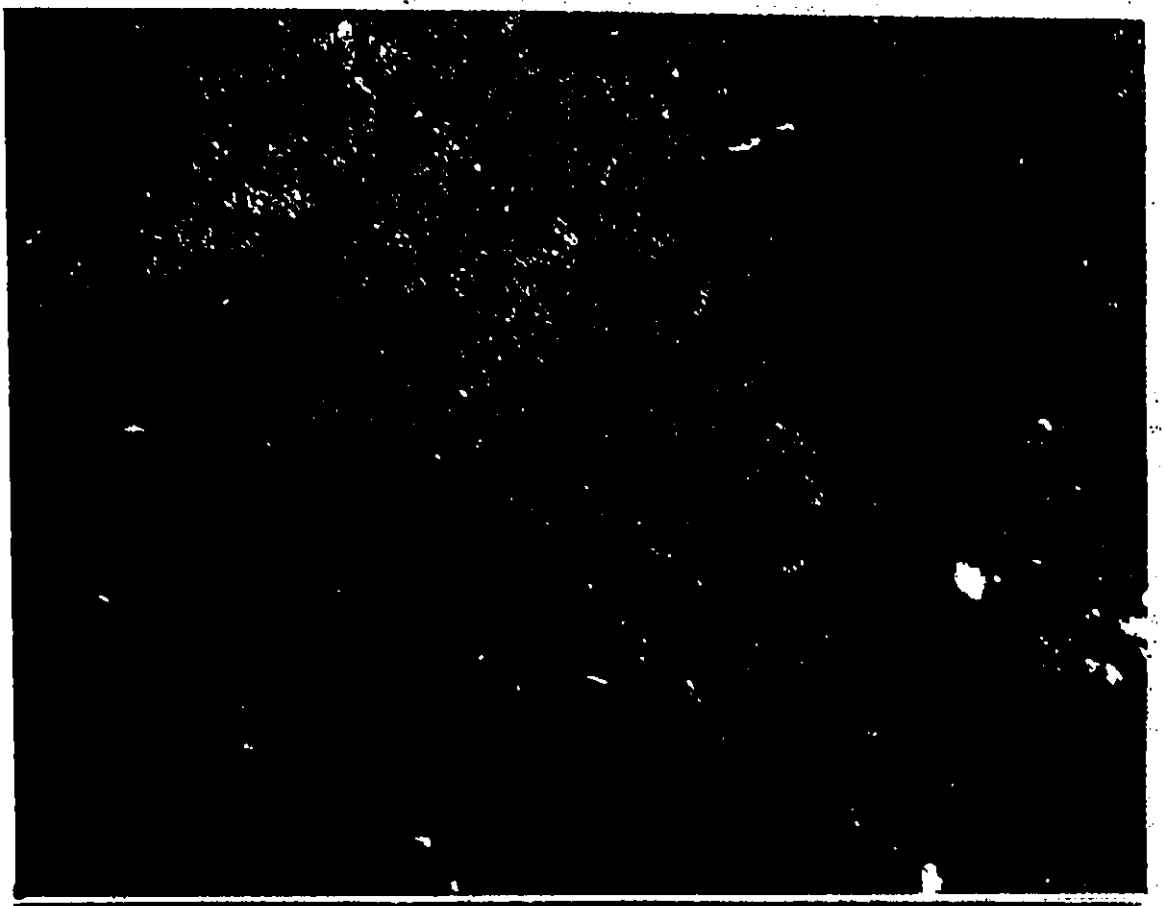
(D) Cold-acclimated,oxytetracycline-treated rat, white fiber, (S-T)*,

The treatment with oxytetracycline affected both types of fibers,red and white, The mitochondria show a generalized disorganization of the cristae with some circular inner membrane. Also a reduction in the number of mitochondria is apparent with a general predominance of the big mitochondria.

Taken at 15000 X magnification with the electron microscope: magnification in printing X 2.

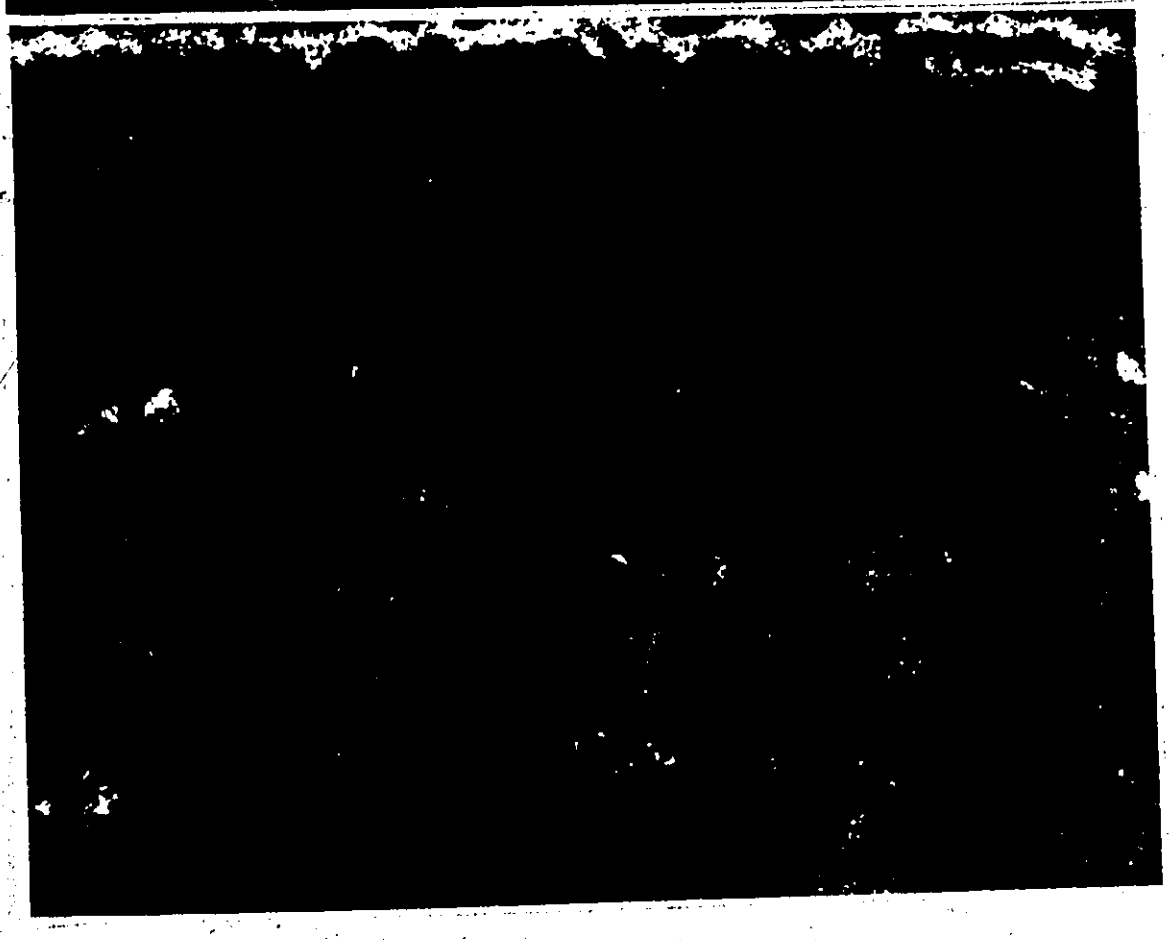
* See legend to Fig.41 for explanation of experimental design.

(A)



(B)

(C)



(D)

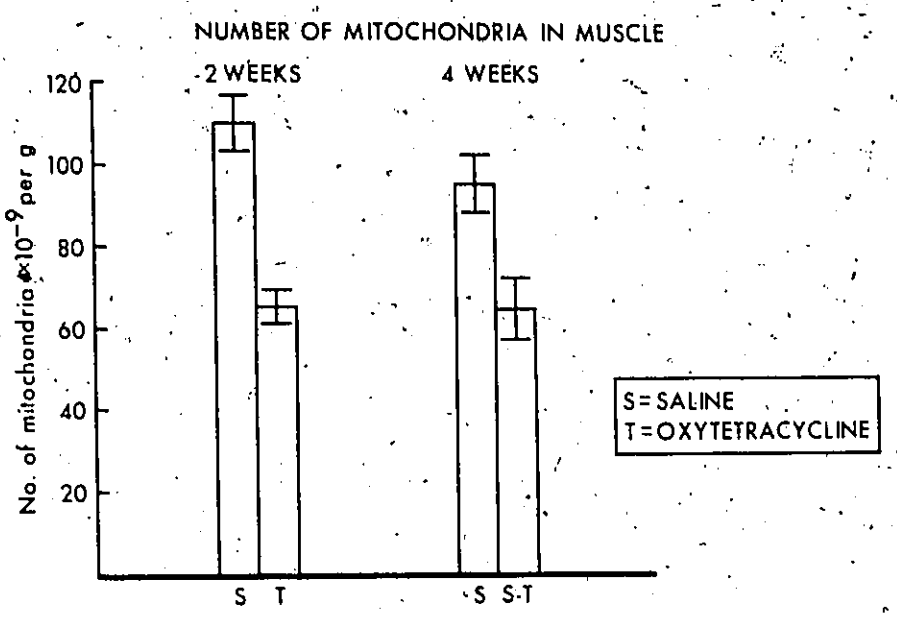


Figure 41. Number of mitochondria from mixed muscle of saline-treated rats (S) and oxytetracycline-treated rats (S-T) after 4 weeks of cold-acclimation. All rats were first acclimated to cold for two weeks. Rats in group S were then injected daily with saline for a further two weeks. Rats in group S-T were injected with oxytetracycline during these two further weeks. As a comparison is also included in the figure (at left side) the number of mitochondria from mixed muscle of saline-treated (S) and oxytetracycline-treated rats (T) from 2 weeks cold-acclimated rats (see Fig 39).

TABLE XIX. Number of mitochondria and biochemical parameters of isolated mitochondria from mixed muscle of saline-treated rats (group S)* and oxytetracycline-treated rats (group S-T)* after 4 weeks of cold-acclimation.

	Group S	Group S-T	P (S vs S-T)
Recovery: mg mitochondrial protein/g muscle	3.69 ± 0.19	3.34 ± 0.52	NS.
Recovery cytochrome oxidase, %	41.74 ± 2.38	36.23 ± 5.40	NS.
Total mitochondrial protein/g of muscle	8.82 ± 0.44	9.27 ± 0.87	NS.
Protein: picograms/mitochondrion	0.0939 ± 0.0021	0.1407 ± 0.0074	<0.005
Nº mitochondria/g of muscle (10^{-9})	94.967 ± 7.180	64.410 ± 7.433	<0.050
Specific activity cytochrome oxidase µatoms O_2/mg prot.min.	16.680 ± 0.644	13.484 ± 0.760	<0.050

...continued

TABLE XIX. (continuation)

	Group S	Group S-T	P (S vs S-T)
N° mitochondria/mg mitochondrial protein (10^{-9})	10.422 ± 0.298	7.148 ± 0.392	<0.005
Cytochrome oxidase µatoms O_2/mitochondrion.min (10^9)	1.569 ± 0.099	1.894 ± 0.131	NS.
Cytochrome oxidase µatoms O_2/g muscle.min.	147.57 ± 1.88	124.21 ± 6.59	<0.050

Means ± SE; n=3

*All rats were first acclimated to cold for two weeks. Rats in Group S were then injected daily with saline for a further two weeks. Rats in Group S-T were injected daily with oxytetracycline during these two further weeks.

treated with oxytetracycline only after two weeks of cold-acclimation had occurred; the number was reduced to the same level as that seen in rats which had been treated with oxytetracycline from the start of cold-acclimation (Figure 41) and to the same level as that seen in untreated warm-acclimated rats (compare Fig. 41 with Fig. 39). The size of the mitochondria increased again in these oxytetracycline-treated cold-acclimated rats to the level seen in warm-acclimated rats, as shown by the high protein content per mitochondrion and the high cytochrome oxidase content per mitochondrion (Table XIX; compare with Table XVIII).

Thus, inhibition of mitochondrial protein synthesis with oxytetracycline can not only prevent the development of the adaptation for nonshivering thermogenesis (enhanced metabolic response to noradrenaline), as shown in Section B, and simultaneously prevent the appearance of the increased number and decreased size of muscle mitochondria (as shown in Section C), but can also cause the reversal of these changes when they have already occurred. Treatment with oxytetracycline of rats in which cold-acclimation has already proceeded for two weeks reverses both the increase in metabolic response to noradrenaline (Section B) and the increase in number and decrease in size of the muscle mitochondria to the levels seen in non-acclimated animals.

Two principal conclusions can be drawn from these results:

1. The increase in number and decrease in size of muscle mitochondria in the cold-acclimated rat is closely

associated with the capacity of that rat for nonshivering thermogenesis.

2. Mitochondrial protein synthesis is necessary for the maintenance of both the changes in muscle mitochondria and the enhanced metabolic response to noradrenaline in cold-acclimated rats which continue to live in the cold.

Section E. MORPHOLOGY AND BIOCHEMICAL PROPERTIES OF MUSCLE MITOCHONDRIA IN COLD-ACCLIMATED RATS DURING DEACCLIMATION.

a) Purpose of the experiment: To find out whether the increase in number and decrease in size of muscle mitochondria in cold-acclimated rats disappears in association with the disappearance of the enhanced metabolic response to noradrenaline during cold-deacclimation. It is known that when a cold-acclimated rat undergoes cold-deacclimation (at room temperature, 25-28°C) the magnitude of the calorigenic response to noradrenaline progressively decreases until it reaches levels similar to those seen in the warm-acclimated rat (Bartunkova et al, 1971; see Fig. 9).

b) Description of the experiment: A total of 56 male rats (112.75 ± 5.10 g) were separated into two groups; one group (warm-acclimated; 22 rats) remained at room temperature during all stages of the experiment and the other group (cold-acclimated; 34 rats) lived at 4°C. Both groups of rats remained at their

temperature of acclimation for 4 weeks. At the end of this period some rats from each group were removed from the cages (referred to as week 0 of cold-deacclimation). In some of them, samples of semitendinosus were removed for the histological and electron microscopic studies, and other rats received an infusion of noradrenaline and the oxygen uptake was measured as indicated under Methods. Other rats were used for the isolation of mitochondria. The remainder of the cold-acclimated rats were transferred at this time to room temperature in order to study deacclimation at week 1 and week 2. At the end of each week rats from the warm-acclimated group and rats from the group undergoing deacclimation were removed and processed similarly to those of week 0. The final number of rats used in each group is indicated in the Tables and Figures.

c) Results and discussion: At week 0 the histochemical and electron microscopic observations revealed the typical characteristics of the muscles in the warm-acclimated rats and in the cold-acclimated rats as have been previously described (Section A). At week 1 of the period of deacclimation it was very difficult, by using the histochemical demonstration of succinic dehydrogenase, to see any differences by comparing the warm-acclimated rats (week 1) with the cold-deacclimated (week 1) and for this reason no electron microscopic observations were attempted. However, at week 2, the histochemical observations were sufficiently clear in the sense that it was observed that the muscles of the cold-deacclimated rats were very similar

to the corresponding muscle from the warm-acclimated rats (week 2). In fact the electron microscope observations reveal no difference at all between the muscles from warm-acclimated and cold-deacclimated at week 2. For this reason no electron micrographs are included for this experiment.

Table XX and Figure 42 show the metabolic response to noradrenaline of these groups of rats. It can be observed that at week 0 the cold-acclimated rats present the normal large increase in oxygen uptake during the infusion of noradrenaline, in comparison with the warm-acclimated rats. This increase disappears slowly with the time which the cold-acclimated rat lived in the warm. At week 2 no significant difference exists between warm-acclimated rats and the cold-deacclimated rats. Together with the disappearance of the enhanced metabolic response to noradrenaline the increased number of mitochondria in the muscle (N° mitochondria/g muscle) also is reduced, reaching at week 2 of deacclimation values close to those of muscle of warm-acclimated rats (Table XXI; Fig. 42). The size of the mitochondria increased again in these cold-deacclimated rats as indicated by the increased protein content per mitochondrion and the increased cytochrome oxidase content per mitochondrion (Table XXI).

Thus, deacclimation to cold reversed the increase in the number of mitochondria, the decrease in size of the mitochondria and the enhancement of the metabolic response to noradrenaline, all of which has been promoted by the long period of cold-acclimation. This result suggests again that the adaptive

TABLE XX. Increase in oxygen uptake* during a 30 min intravenous infusion of noradrenaline in cold-acclimated rats undergoing deacclimation for 1 or 2 weeks and in warm-acclimated rats.

	Warm-Acclimated	Cold-Acclimated	P (warm vs cold)
WEEK 0.	53.30 ± 3.51(n 3)	93.88 ± 3.56(n 4)	<0.001
WEEK 1.	43.20 ± 6.16(n 3)	67.43 ± 3.93(n 4)	<0.020
WEEK 2.	46.13 ± 3.68(n 3)	51.00 ± 4.15(n 4)	NS.
P(WEEK 0 vs WEEK 1)	NS.	<0.005	
P(WEEK 0 vs WEEK 2)	NS.	<0.001	

Means ± SE.

*ml O_2/100 cm^2.30 min.

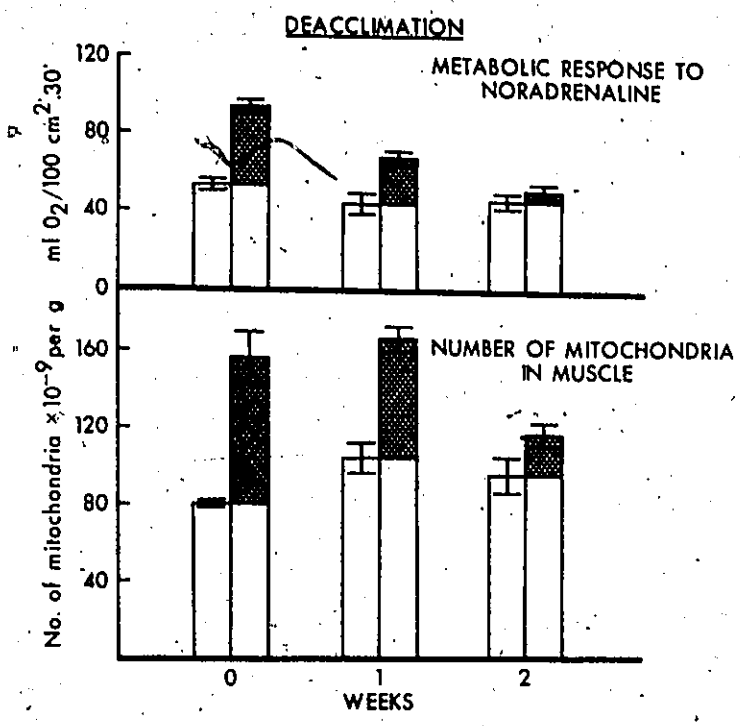


Figure 42. Increase in oxygen uptake during a 30 minutes intravenous infusion of noradrenaline (upper part) and number of mitochondria of mixed muscles (lower part) in cold-acclimated rats (right bar) undergoing deacclimation for 1 or 2 weeks and in warm-acclimated rats (left bar).

TABLE XXI. Number and biochemical parameters of muscle mitochondria in cold-acclimated rats undergoing deacclimation for 1 or 2 weeks and in warm-acclimated rats.

	Week	Warm-Acclimated	Cold-Acclimated	P (warm vs cold)
Recovery:mg mitochondrial protein/g muscle	0	3.44 ± 0.49	2.70 ± 0.32	NS.
	1	4.89 ± 0.46	3.48 ± 0.49	NS.
	2	3.63 ± 0.21	3.68 ± 1.09	NS
Recovery cytochrome oxidase, %	0	34.58 ± 2.36	22.64 ± 3.18	<0.050
	1	40.05 ± 5.04	21.36 ± 1.58	<0.025
	2	28.28 ± 1.12	24.61 ± 3.93	NS.
Total mitochondrial protein/g of muscle	0	10.063 ± 0.565	12.186 ± 1.586	NS.
	1	12.325 ± 0.613	16.232 ± 1.240	<0.050
	2	12.829 ± 0.484	14.512 ± 2.160	NS.
Protein: picograms/mitochondrion	0	0.1247 ± 0.0040	0.0777 ± 0.0038	<0.005
	1	0.1192 ± 0.0039	0.0976 ± 0.0037	<0.020
	2	0.1369 ± 0.0181	0.1252 ± 0.0143	NS.
N° mitochondria/g of muscle (10^{-9})	0	80.562 ± 2.131	155.917 ± 14.243	<0.010
	1	103.744 ± 7.496	165.832 ± 6.653	<0.005
	2	95.357 ± 9.210	115.410 ± 7.008	NS.

...continued

TABLE XXI. (continuation)

	Week	Warm-Acclimated	Cold-Acclimated	P (warm vs cold)
Specific activity cytochrome oxidase µatoms O_2/mg protein.min.	0	18.373 ± 2.190	16.280 ± 1.700	NS.
	1	14.536 ± 0.443	11.440 ± 0.756	<0.025
	2	14.023 ± 1.329	12.423 ± 1.533	NS.
Nº mitochondria/mg mitochondrial protein (10^{-9})	0	8.036 ± 0.269	12.929 ± 0.654	<0.020
	1	8.405 ± 0.270	10.277 ± 0.398	<0.020
	2	7.476 ± 0.858	8.197 ± 0.843	NS.
Cytochrome oxidase µatoms O_2/mitochondrion. min (10^{92})	0	2.279 ± 0.220	1.255 ± 0.092	<0.020
	1	1.735 ± 0.098	1.117 ± 0.091	<0.010
	2	1.888 ± 0.079	1.512 ± 0.065	<0.025
Cytochrome oxidase µatoms O_2/g múscle. min.	0	177.56 ± 11.82	192.99 ± 3.60	NS.
	1	179.83 ± 11.88	184.62 ± 19.19	NS.
	2	179.78 ± 17.01	173.70 ± 5.07	NS.

Means ± SE. (n=3)

changes observed in the muscle mitochondria of cold-acclimated rats are closely associated with the nonshivering thermogenesis process because they disappear in parallel with the adaptation for nonshivering thermogenesis when the stimulus which promotes the adaptive state is no longer present.

Section F. MORPHOLOGY AND BIOCHEMICAL PROPERTIES OF MUSCLE MITOCHONDRIA IN COLD-ACCLIMATED RATS IN WHICH DEACCLIMATION IS ACCELERATED BY REMOVAL OF INTERSCAPULAR BROWN ADIPOSE TISSUE.

a) Purpose of the experiment: To find out whether the more rapid rate of deacclimation to cold produced by removal of the interscapular brown adipose tissue (Himms-Hagen, 1969, 1970) is accompanied by a more rapid reversal of the changes in muscle mitochondria induced by cold-acclimation.

As was indicated in Chapter I of this thesis, one of the purposes of this research is to find additional support for the postulated endocrine role of brown adipose tissue during cold-acclimation (Himms-Hagen, 1969, 1970). It is therefore another purpose of this experiment to find out whether removal of the interscapular brown adipose tissue causes measurable changes in other tissues (specifically in skeletal muscle).

b) Description of the experiment: A total of 66 male rats (127 ± 4.80 g) were used in this experiment. They were divided into 2 major groups; warm-acclimated rats (30 rats) and cold-acclimated rats (36 rats). Both groups remained at their

respective temperature of acclimation for a period of 4 weeks, at the end of which all the rats were transferred to room temperature. Rats of both groups were removed from the cages and operated, as described under Methods. The rats in which interscapular brown adipose tissue (IBAT) was removed were designed: Cold-IBAT and Warm-IBAT, depending on whether they had been cold-acclimated or warm-acclimated. The rats in which IBAT was left intact were designated: Cold, Sham and Warm, Sham. Some rats from each of the 4 groups were processed on the same day of the operation (referred to as day 0); some of them received an infusion of noradrenaline in order to measure the metabolic response to noradrenaline, others were used for the histological and electron microscopic studies and others used for the collection of 10 g of mixed muscles in order to isolate mitochondria by the protease digestion method as previously described.

The remainder of the operated rats (which were now living in the warm) remained in individual cages for 2 extra days, (referred to as day 2) at the end of which some of them were removed and processed in the same way as the rats of day 0. The remainder of the operated rats (-IBAT and Sham) remained in the warm for a total of 5 days (referred as day 5) at the end of which they were removed and processed in the same way as the rats from day 0 and day 2. The number of rats used in the different parts of the experiment are indicated in the Tables and Figures.

c) Results and discussion: The histochemical observations showed that at day 0 all the changes described for the cold-acclimated rats were present in comparison with the warm-acclimated rats. However, at day 2 and day 5 it was very difficult to see any major changes in the fiber characteristics of the rats from the group Cold-IBAT in comparison with their counterpart: Cold, Sham. For this reason no electron microscopic observations were attempted in this experiment. It was supposed that the difference (if it existed) was so small that the electron microscopic observation would not be able to detect it with clarity. Therefore the results of this experiment are based only in the counting of isolated mitochondria and the measurement of several biochemical parameters in these mitochondria.

The enhanced metabolic response to noradrenaline diminished more rapidly in the rats from which the IBAT had been removed (Fig. 43), as had been observed previously (Himms-Hagen, 1969, 1970). By day 5 the metabolic response reached values approaching those seen in warm-acclimated rats (Fig. 43; Table XXIII).

The increased number of mitochondria also decreased more rapidly in the rats without their IBAT (Fig. 43) reaching a value approximately midway between that of sham-operated cold-acclimated rats and that of warm-acclimated rats by day 5. The size of the mitochondria also tended to increase more rapidly in the rats without IBAT, as indicated by the changes in protein content per mitochondrion and cytochrome oxidase content

per mitochondrion, although the differences between the two groups (Cold-IBAT and Cold, Sham) are not statistically significant. The changes measured in the muscle mitochondria (decrease in number and increase in size) appear to lag behind somewhat the changes occurring in the enhanced metabolic response to noradrenaline. They are, nevertheless, occurring more rapidly than during normal deacclimation to cold (compare Fig. 43 with Fig. 42). (See Table XXII).

The following conclusions can be drawn from this experiment:

1. The increase in number and decrease in size of muscle mitochondria is once again seen to be closely associated with the capacity of the cold-acclimated rat for non-shivering thermogenesis (enhanced metabolic response to noradrenaline).

2. Since removal of the interscapular brown adipose tissue causes a measurable change in muscle (reduction in the increased number of mitochondria, an increase demonstrated to be closely associated with the adaptation for nonshivering thermogenesis) this result can be taken as providing further support for a role of interscapular brown adipose tissue in maintaining the adaptation for nonshivering thermogenesis in muscle.

It can be speculated that the postulated factor secreted by the interscapular brown adipose tissue promotes the development and maintenance of the adaptive state in muscle via an action which results in an increase in the number and decrease in the

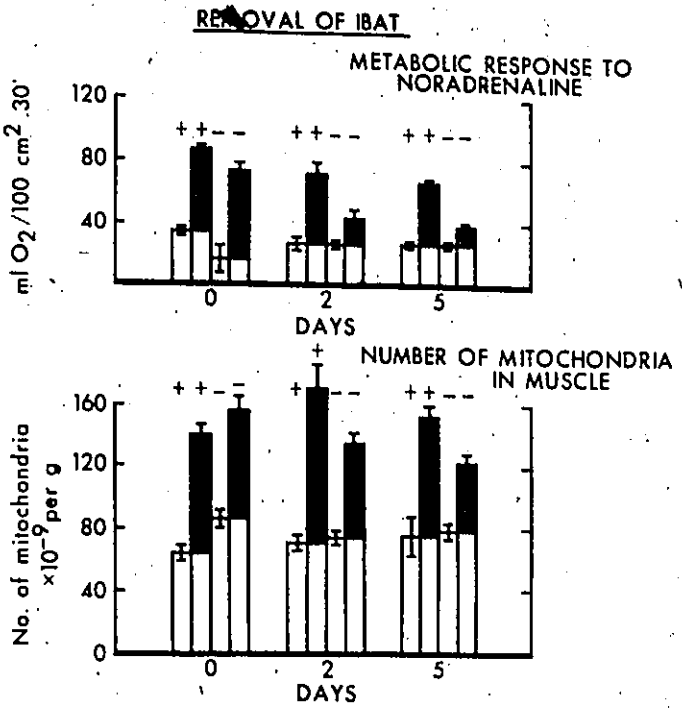


Figure 43. Effect of removal of interscapular brown adipose tissue (IBAT) upon total increase in oxygen uptake during a 30 minutes intravenous infusion of noradrenaline (upper part) and number of mitochondria (lower part) of warm-acclimated (left bar) and cold-acclimated rats (right bar). From day 0, day of the operation, all rats were kept at room temperature. (+ means sham-operated animals; - refers to rats from which the interscapular brown adipose tissue was removed on day 0).

TABLE XXII. Effect of the removal of interscapular brown adipose tissue upon number and biochemical parameters of muscle mitochondria of warm-acclimated and cold-acclimated rats*

	Day;	Operation	Warm-acclimated	Cold-Acclimated*	P (warm vs cold)
Recovery: mg mitochondrial protein/g muscle	Day 0	SHAM †	3.26 ± 0.32	2.71 ± 0.72	NS.
		-IBAT †	3.31 ± 0.34	2.72 ± 0.28	NS.
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 2	SHAM	2.98 ± 0.61	3.06 ± 0.37	NS.
		-IBAT	2.91 ± 0.31	3.25 ± 0.46	NS.
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 5	SHAM	4.11 ± 0.70	3.26 ± 0.43	NS.
		-IBAT	3.66 ± 0.52	2.82 ± 0.50	NS.
		P(SHAM vs -IBAT)	NS.	NS.	
Recovery: cytochrome oxidase %	Day 0	SHAM	37.63 ± 4.59	26.79 ± 5.62	NS.
		-IBAT	31.21 ± 4.42	25.53 ± 3.09	NS.
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 2	SHAM	31.07 ± 4.69	28.73 ± 3.45	NS.
		-IBAT	28.64 ± 2.68	33.42 ± 0.77	NS.
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 5	SHAM	41.79 ± 2.73	30.02 ± 3.30	<0.05
		-IBAT	32.63 ± 3.29	26.18 ± 1.99	NS.
		P(SHAM vs -IBAT)	NS.	NS.	

... continued

TABLE XXII (continued)

	Day;	Operation	Warm-acclimated	Cold-Acclimated	P (warm vs cold)
Total mitochondrial protein/g of muscle	Day 0	SHAM	8.94 ± 1.37	9.83 ± 0.74	NS.
		-IBAT	10.82 ± 0.99	10.72 ± 0.39	NS.
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 2	SHAM	9.64 ± 1.25	10.65 ± 0.08	NS.
		-IBAT	10.18 ± 0.69	9.72 ± 1.35	NS.
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 5	SHAM	9.72 ± 1.12	10.80 ± 0.23	NS.
		-IBAT	11.10 ± 0.51	10.82 ± 1.67	NS.
		P(SHAM vs -IBAT)	<0.050	NS.	
Protein: picograms/ mitochondrion	Day 0	SHAM	0.1362 ± 0.0110	0.0698 ± 0.0037	<0.005
		-IBAT	0.1256 ± 0.0032	0.0690 ± 0.0021	<0.001
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 2	SHAM	0.1338 ± 0.0097	0.0630 ± 0.0055	<0.005
		-IBAT	0.1417 ± 0.0163	0.0767 ± 0.0066	<0.025
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 5	SHAM	0.1337 ± 0.0100	0.0707 ± 0.0012	<0.005
		-IBAT	0.1435 ± 0.0080	0.0872 ± 0.0095	<0.020
		P(SHAM vs -IBAT)	NS.	NS.	

...continued

TABLE XXII (continuation)

	Day; Operation	Warm-acclimated	Cold-Acclimated	P (warm vs cold)
Nº mitochondria/ g of muscle (10^{-9})	Day 0 SHAM	64.649 ± 5.077	140.878 ± 7.578	<0.005
	-IBAT	85.989 ± 6.483	156.157 ± 10.600	<0.005
	P(SHAM vs -IBAT)	NS.	NS.	
	Day 2 SHAM	71.442 ± 5.064	171.570 ± 15.907	<0.005
	-IBAT	73.459 ± 5.910	125.494 ± 8.148	<0.010
	P(SHAM vs -IBAT)	NS.	NS.	
	Day 5 SHAM	74.756 ± 13.770	152.972 ± 5.925	<0.010
	-IBAT	77.875 ± 5.645	122.898 ± 5.614	<0.005
	P(SHAM vs -IBAT)	NS.	< 0.025	
Specific Activity Cytochrome Oxidase µatoms O_2/mg protein. min.	Day 0 SHAM	19.855 ± 1.793	17.225 ± 0.919	NS.
	-IBAT	19.087 ± 1,064	17.508 ± 1.148	NS.
	P(SHAM vs -IBAT)	NS.	NS.	
	Day 2 SHAM	18.079 ± 1.954	16.088 ± 1.053	NS.
	-IBAT	18.817 ± 1.299	16.754 ± 2.380	NS.
	P(SHAM vs -IBAT)	NS.	NS.	
	Day 5 SHAM	16.096 ± 0.463	16.338 ± 1.451	NS.
	-IBAT	14.379 ± 0.610	16.400 ± 0.914	NS.
	P(SHAM vs -IBAT)	NS.	NS.	

...continued

TABLE XXII. (continuation)

	Day;	Operation	Warm-acclimated	Cold-Acclimated	P (warm vs cold)
Nº mitochondria/ mg mitochondrial protein (10^{-9})	Day 0	SHAM	7.439 ± 0.614	14.419 ± 0.763	<0.005
		-IBAT	7.971 ± 0.201	14.535 ± 0.456	<0.001
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 2	SHAM	7.555 ± 0.572	16.106 ± 1.427	<0.010
		-IBAT	7.236 ± 0.784	13.226 ± 1.144	<0.020
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 5	SHAM	7.567 ± 0.585	14.157 ± 0.242	<0.001
		-IBAT	7.019 ± 0.426	11.822 ± 1.514	NS.
		P(SHAM vs -IBAT)	NS.	NS.	
Cytochrome oxidase µatoms O_2/ mitochondrion.min (10^9)	Day 0	SHAM	2.666 ± 0.022	1.198 ± 0.055	<0.001
		-IBAT	2.394 ± 0.118	1.203 ± 0.050	<0.001
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 2	SHAM	2.399 ± 0.228	1.009 ± 0.083	<0.005
		-IBAT	2.655 ± 0.313	1.255 ± 0.068	<0.020
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 5	SHAM	2.149 ± 0.158	1.156 ± 0.113	<0.010
		-IBAT	2.060 ± 0.123	1.412 ± 0.101	<0.020
		P(SHAM vs -IBAT)	NS.	NS.	

...continued

TABLE XXII (continuation)

	Day;	Operation	Warm-acclimated	Cold-Acclimated	P (warm vs cold)
Cytochrome oxidase	Day 0	SHAM	172.085 ± 12.061	169.499 ± 16.831	NS.
		-IBAT	204.389 ± 7.718	188.560 ± 20.376	NS.
µatoms O_2/g muscle.min.		P(SHAM vs-IBAT)	NS.	NS.	
	Day 2	SHAM	170.525 ± 16.932	171.148 ± 9.861	NS.
		-IBAT	189.997 ± 6.395	156.439 ± 3.667	<0.020
		P(SHAM vs -IBAT)	NS.	NS.	
	Day 5	SHAM	156.451 ± 19.056	176.557 ± 16.420	NS.
		-IBAT	159.028 ± 1.616	174.647 ± 19.567	NS.
		P(SHAM vs -IBAT)	NS.	NS.	

Means ± SE; n = 3

*From day 0, day of the operation, all rats were kept at room temperature.

†SHAM means sham-operated animals; -IBAT refers to rats from which the interscapular brown adipose tissue was removed on day 0.

TABLE XXIII. Effect of removal of interscapular brown adipose tissue upon total increase in oxygen uptake* during a 30 min intravenous infusion of noradrenaline of warm-acclimated and cold-acclimated rats§.

Group		Warm-Acclimated	Cold-Acclimated§	P (warm vs cold)
Day 0	SHAM†	34.03 ± 2.48 (n=3)	87.42 ± 2.21 (n=3)	<0.001
	-IBAT†	17.33 ± 9.31 (n=3)	72.83 ± 5.81 (n=3)	<0.001
	P(SHAM vs -IBAT)	NS.	NS.	
Day 2	SHAM	26.00 ± 4.44 (n=3)	70.75 ± 9.25 (n=2)	<0.020
	-IBAT	24.70 ± 2.70 (n=3)	43.50 ± 5.50 (n=2)	<0.050
	P(SHAM vs -IBAT)	NS.	NS.	
Day 5	SHAM	26.63 ± 0.49 (n=3)	66.53 ± 2.34 (n=3)	<0.001
	-IBAT	25.60 ± 1.31 (n=3)	38.07 ± 2.32 (n=3)	<0.010
	P(SHAM vs -IBAT)	NS.	<0.001	

Mean ± SE.

*ml O_2/100 cm^2.30 min.

†-SHAM means sham-operated animals; -IBAT refers to rats from which the interscapular brown adipose tissue was removed on day 0.

§-From day 0, day of the operation, all rats were kept at room temperature.

in the size of the mitochondria, an alteration which possibly involves an altered mitochondrial protein synthesis.

CHAPTER V. CONCLUSION

The principal aim of the experiments described in this thesis was to answer the question: Does acclimation to cold produce adaptive changes in muscle mitochondria ?

In order to answer this question a comparison of both red and white muscles of cold-acclimated rats with both red and white muscles of warm-acclimated rats was performed in the following ways:

- Electron microscopy of red and white muscles.
- Fiber characteristics and distribution in red and white muscles
- Measurement of number, size and various enzyme activities of mitochondria isolated from muscle.

These procedures were carried out on rats that were undergoing acclimation to cold, or fully acclimated to cold, or undergoing deacclimation to cold. In addition, conditions were studied in which the normal acclimation process was modified: by prevention of the acclimation to cold with oxytetracycline, by reversal of the acclimation to cold with oxytetracycline, by acceleration of the deacclimation to cold by removal of the interscapular brown adipose tissue. The last type of experiment was hoped to give satisfaction to the subsidiary aim of this research which is to provide further evidence for an endocrine function of brown adipose tissue in the development and maintenance of the cold-acclimated state.

In these experiments, a marked difference between mitochondria of muscles of cold-acclimated rats and mitochondria of muscles of warm-acclimated control rats has been seen. This is the first time such a difference has been observed. The principal differences seen are:

1. An increase in the number and a reduction in the size of the mitochondria in both red and white muscle.
2. An increase in the proportion of red fibers and an apparent transformation of white fibers into fibers which appear more like intermediate fibers.

The first indication that these changes are related to nonshivering thermogenesis can be drawn from the fact that they were observed as early as two weeks of cold-acclimation (Section C of Results, Chapter IV) just when nonshivering thermogenesis had partially developed, and as late as four months (Section A of Results, Chapter IV) when nonshivering thermogenesis had been fully developed. In other words, the described changes parallel the development and maintenance of the adaptation for nonshivering thermogenesis.

In spite of the increase in number and decrease in size of the mitochondria of muscle of cold-acclimation rats, the mitochondrial mass per gram of muscle is unchanged. This indicates an altered distribution of the mitochondrial mass into smaller units and suggests the existence of a mechanism which may modify the properties of these mitochondria in such a way as to alter their function during the development and maintenance of nonshivering thermogenesis.

It should be noted also that the changes observed in muscle mitochondria of cold-acclimated rats are different from the changes known to occur in brown adipose tissue mitochondria of cold-acclimated rats. This, together with the lack of significant changes in liver mitochondria during cold-acclimation, suggests that adaptive changes take place only in those organs where nonshivering thermogenesis take place; namely skeletal muscle and brown adipose tissue and, that the nature of the adaptation is different in these two tissues.

That the increase in number and decrease in size of muscle mitochondria in the cold-acclimated rat is associated with the development of the adaptation for nonshivering thermogenesis is shown by the fact that inhibition of the development of the adaptation by oxytetracycline is accompanied by inhibition of the appearance of the increase in number of muscle mitochondria. The inhibition by oxytetracycline is an indication that an active mitochondrial protein synthesis is necessary for the increase in number and decrease in size of muscle mitochondria to occur during cold-acclimation. Moreover, inhibition of mitochondrial protein synthesis with oxytetracycline can not only prevent the development of the adaptation for nonshivering thermogenesis, and simultaneously prevent the appearance of the increased number and decreased size of muscle mitochondria, but can also cause the reversal of these changes when they have already occurred together with the reversal of the enhanced metabolic response

to noradrenaline. This finding indicates that the adaptive changes that take place in muscle mitochondria during cold-acclimation are closely associated with the capacity of the rat for nonshivering thermogenesis and that mitochondrial protein synthesis is necessary for maintenance of both the changes in muscle mitochondria and the enhanced metabolic response to noradrenaline in cold-acclimated rats which continue to live in the cold.

Another indication that the adaptive changes in muscle mitochondria observed in the muscle of cold-acclimated rats are associated with the nonshivering thermogenesis process can be found in the fact that deacclimation to cold, which is known to slowly reverse the adaptive state, also reverses in parallel the changes in muscle mitochondria indicating that like other adaptive changes in muscle mitochondria they disappear when the stimulus which promotes the adaptive state is no longer present. Acceleration of the deacclimation process by removal of interscapular brown adipose tissue also accelerates the disappearance of the adaptive changes in muscle mitochondria in parallel with the more rapid disappearance of the enhanced metabolic response to noradrenaline, indicating once more the close association between the adaptive state and the increase in number and decrease in size of the muscle mitochondria in the cold-acclimated rat.

Thus, it is possible to conclude that the adaptive changes of muscle mitochondria in the cold-acclimated rat are part of the mechanism of nonshivering thermogenesis, but

the way in which they participate in or control the mechanism itself is not known.

The changes in muscle mitochondria during cold-acclimation differ from adaptive changes in muscle mitochondria induced by other stimuli which alter muscle function. Training, for example, produces an increase in the red fibers and an increased number of larger mitochondria with more densely packed cristae; training increases the capacity of the muscle to obtain energy by oxidation of substrates. In contrast, cold-acclimation does not alter the capacity of muscle to oxidize substrates, as indicated by the unaltered mitochondrial mass. Cold-acclimation may be presumed to alter the way in which the use of the capacity that is already there is regulated; this is reflected in the altered distribution of mitochondrial material.

The subsidiary aim of this research, namely, to provide further evidence for an endocrine role of interscapular brown adipose tissue has also in part been satisfied. The removal of the interscapular brown adipose tissue caused the expected change in muscle, i.e. a reduction in the increased number of mitochondria. Therefore, as the increase in number of mitochondria is closely associated with the adaptation for nonshivering thermogenesis this result can be taken as providing further support for a role of brown adipose tissue in maintaining the adaptation for nonshivering thermogenesis in muscle. It can be speculated that the postulated factor secreted by the interscapular brown adipose tissue promotes

the development and maintenance of the adaptive state in muscle via an action which results in an increase in the number and decrease in the size of the mitochondria, an alteration which possibly involves an altered mitochondrial protein synthesis.

Two previous findings in this laboratory have suggested a special role for mitochondrial protein synthesis in acclimation to cold. First, a decreased half-life of certain mitochondrial proteins occurs in skeletal muscle and brown adipose tissue of cold-acclimated rats; nonshivering thermogenesis is known to occur in both these tissues. No such decrease occurs in liver or kidney, tissues in which nonshivering thermogenesis does not occur (Bukowiecki and Himms-Hagen, 1971). This finding adds to the conclusion that acclimation to cold causes a change in the rate of synthesis and/or in the amount of certain mitochondrial proteins and that this change might be associated with the development of the capacity for nonshivering thermogenesis. Second, inhibition of mitochondrial protein synthesis with oxytetracycline inhibits the development of the adaptation (Himms-Hagen, 1971). This latter finding was confirmed in the course of this research and extended to show that the maintenance of the adaptation can also be inhibited by the antibiotic.

The mechanism by which the formation of more but smaller mitochondria occurs in muscle of cold-acclimated rats is not known. The participation of mitochondrial protein synthesis is suggested by the two findings outlined above. Three pos-

sible mechanisms can be suggested:

(i) division of existing mitochondria
(ii) de novo synthesis of different mitochondria
(iii) both (i) and (ii) occurring simultaneously or sequentially

The results of the present research do not permit a distinction to be made among the proposed mechanisms. It seems unlikely that the first mechanism is of major importance. The electron microscopic observations performed in the present study failed to reveal any partitioned mitochondria. Moreover, most of the new mitochondria were found in the interior of the muscle fibers where few mitochondria were seen before the period of cold-acclimation. Division has been postulated as a mechanism of mitochondrial proliferation (Tandler et al., 1969; Tandler and Hoppel, 1972) and partitioned mitochondria have been commonly observed in conditions in which proliferation of mitochondria is known to occur. However, other authors question this interpretation of the occurrence of partitioned mitochondria and suggest that these represent fused rather than dividing mitochondria (Dallman and Goodman, 1971).

Studies directed toward biogenesis of mitochondria in intact cells have focused in large part on unicellular organisms such as yeast, in which release from glucose repression, or anaerobiosis, or a promitochondrial state (Plattner and Schatz, 1969; Schtz, 1970), leads to rapid changes involving

appearance of recognizable mitochondria and restoration of respiratory activity. The mode of mitochondrial formation has been so far directly investigated only in Neurospora crassa. By using a choline-requiring mutant of this organism, Luck (1965) has obtained evidence which strongly suggests that mitochondria are formed by growth and division of prexisting mitochondria. In Tetrahymena pyriformis Parsons and Rustad (1968) also found that the mode of mitochondrial formation follows a pattern compatible with this suggestion.

In animal cells no comparable evidence presently exists regarding the mode of mitochondrial formation although several mammalian systems for the study of mitochondrial formation have been used. These include the proliferation of kidney mitochondria induced by unilateral nephrectomy (Mize and Worthen, 1972) and the proliferation of kidney mitochondria induced by cell damage provoked by administration of egg albumin (Pugh, 1972). The best system appears to be the formation of mitochondria in HeLa cells (Storrie and Attardi, 1973). Storrie and Attardi (1973) suggest that mammalian mitochondria replicate by a random insertion of components into the membranes and subsequent division (mechanism (iii) above). This suggestion is based upon the finding that no significant diminution in the number of cytochrome oxidase-containing mitochondria occurred during four cell generations of HeLa cells in which no mitochondrial protein synthesis could occur because the cells were grown in the presence of chloramphenicol; each mitochondrion nevertheless contained less cytochrome oxidase in each succeeding generation.

No studies of the replication of skeletal muscle mitochondria appear to have been made. Studies of muscle mitochondria have concentrated on detecting changes in form and number but have not looked at the mode of formation of the altered mitochondria although partitioned mitochondria have been observed in muscle fibers that are regenerating after injury (Reznik and Hansen, 1969).

An interesting study of muscle mitochondria which is relevant to the subject of this thesis is contained in a study of a patient who presented with severe hypermetabolism of nonthyroid origin (Luft et al, 1962). Electron microscopic examination of the patient's skeletal muscle revealed an increased number of mitochondria in the perinuclear areas of the fibers. The mitochondria were of extremely variable size and contained a vast amount of densely packed cristae. Also other structural changes of the mitochondria were observed, such as tubular inner structures instead of cristae, lack of opaque bodies, presence of dense, regularly layered cores and occurrence of cylindrical mitochondria surrounded by concentric sheaths. The biochemical studies with isolated mitochondria revealed a loosely coupled state of the oxidative phosphorylation in the patient's mitochondria, characterized by a nearly maximal rate of respiration in the absence of phosphate acceptor, but an essentially normal extent of phosphorylation when phosphate acceptor was present. The sensitivity of the respiration to oligomycin was only partial. The mitochondria exhibited a high Mg^{++}-dependent ATPase

activity that was only slightly stimulated by dinitrophenol. Also a large increase in total mitochondrial protein was found with an increased cytochrome oxidase activity per unit of mitochondrial protein. The authors concluded that the hypermetabolic state of the patient was caused by a defect in the mitochondrial enzyme organization, resulting in a severely lowered capacity for respiratory control. Thus, these muscle mitochondria appear to be permanently loosely coupled: this results in a permanent elevation of metabolic rate and indicates that a modification of mitochondrial structure can cause the appearance of a loosely coupled state. The modification of mitochondrial structure which can be seen to occur in the muscle of the cold-acclimated rat cannot be of the same kind as that seen in the patient described above since it does not result in a permanent elevation of metabolic rate but only evokes the capacity to elevate metabolic rate when appropriately stimulated.

Before the studies reported in this thesis no differences in structure or function of mitochondria of muscle of cold-acclimated rats had been reported. Therefore these studies, together with the studies of Bukowiecki and Himms-Hagen (1971) which reported a decrease in the half-life of some mitochondrial proteins in muscle and brown adipose tissue, are the first to indicate that major changes take place in the muscle mitochondria of cold-acclimated rats. It is expected that the approach used in this research can give further information which could lead to an explanation of the process by which

the change from shivering thermogenesis to nonshivering thermogenesis occurs in the muscle mitochondria of cold-acclimated rats. An obvious way of continuing this study will be a more extensive research into other biochemical parameters of the altered mitochondria. The isolated mitochondria can be investigated with regard to some basic properties relevant to their functional state, such as respiration, phosphorylation, respiratory control and ATPase activity and it will be necessary to obtain a more detailed enzymic profile of these altered mitochondria in order to elucidate the way in which they are altered and how they participate in the process of nonshivering thermogenesis.

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