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MUSCLE MITOCHONDRIAL CALCIUM TRANSPORT IN THE COLD-ACCLIMATED RAT

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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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August, 1976

ACKNOWLEDGMENTS

I would like to thank Dr. Jean Himms-Hagen for her advice and many helpful discussions during the course of this research. The Medical Research Council of Canada is also acknowledged for awarding me a studentship that provided financial support for my studies. Finally, in a very special way, I thank my wife Deborah for her encouragement and typing of the thesis.

SUMMARY

A rat placed in a cold-environment is able to produce heat and maintain its body temperature by shivering. After living in the cold for about a month, the rat undergoes an adaptive change allowing it to maintain its body temperature without shivering. The rat is then said to be cold-acclimated and the term nonshivering thermogenesis is used to describe the process by which it can increase its heat production.

The mechanism of heat production during shivering is similar to that of light exercise. However, the nature and biochemical mechanism of nonshivering thermogenesis is not completely understood. It is known that nonshivering thermogenesis is related to an enhanced calorigenic response to noradrenaline secreted by the animal and that the process takes place largely in the skeletal muscles.

Recent findings in this laboratory have shown that cold-acclimation is related to morphological and biochemical changes in the skeletal muscle mitochondria. These mitochondria become more numerous but smaller in size in the cold-acclimated rat, with the total mitochondrial mass remaining unchanged. An increased turnover of certain groups of mitochondrial proteins is seen. Biochemical differences include increased rates of state 3 respiration, and increased amounts of the enzymes α-glycerophosphate dehydrogenase and adenine nucleotide translocase. Although these findings show that the muscle mitochondria undergo changes associated with cold-acclimation, they have not provided an explanation for the biochemical mechanism of nonshivering thermogenesis.

A possible mechanism of nonshivering thermogenesis in muscle might involve a noradrenaline-induced increase in the recycling of calcium across the mitochondrial inner membrane, since mitochondrial calcium uptake is an energy-linked process, and this would provide a method of increasing respiration and thus heat production in the cold-acclimated rat.

The purpose of the research reported in this thesis was to compare various aspects of calcium transport in muscle mitochondria isolated from cold-acclimated rats and warm-acclimated controls. Differences in calcium transport, if found, might then give a clue to the biochemical mechanism of nonshivering thermogenesis.

The following aspects of mitochondrial calcium transport were compared in warm-acclimated and cold-acclimated rats:

(1) Calcium contents of isolated mitochondria.

(2) Effects of added calcium on mitochondrial respiration.

(3) Measurement of the time course and initial rates of calcium uptake.

(4) Measurement of the time course of spontaneous calcium release and release induced by various physiological agents.

(5) Measurement of the number and affinity of respiration-inhibited high affinity and low affinity calcium binding sites.

No change was found in the calcium contents of isolated muscle mitochondria or in the number and affinity of calcium binding sites. Small increases were observed in the calcium-stimulated respiration rate in mitochondria from cold-acclimated rats with some respiratory substrates. The initial rate of calcium uptake was elevated in mitochondria from cold-acclimated rats. Spontaneous release of calcium from mitochondria was also increased as a result of cold-acclimation. The release of calcium induced

by added sodium ion was the same in mitochondria from cold-acclimated rats and controls.

The results suggest that one of the adaptive changes that muscle mitochondria undergo during cold-acclimation is a change in the calcium transport system such that calcium is taken up and released at a greater rate. The differences observed, however, were small and so it is unlikely that calcium recycling alone could account for the large noradrenaline-induced enhancement of metabolic rate seen in the cold-acclimated rat. Further research is thus required to elucidate completely the biochemical mechanism of non-shivering thermogenesis in skeletal muscle.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BAT	brown adipose tissue
CA	cold-acclimated
cyclic AMP	adenosine 3', 5'-monophosphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(aminoethylether) N,N'-tetraacetic acid
FFA	free fatty acids
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IBAT	interscapular brown adipose tissue
NST	nonshivering thermogenesis
PEP	phosphoenolpyruvate
PGE_1	prostaglandin E_1
RR	ruthenium red
Tris	trishydroxymethylaminomethane
WA	warm-acclimated

CHAPTER I: INTRODUCTION

A rat exposed to a cold environment is able to maintain its body temperature by increasing its metabolic rate through shivering. If the rat remains in the cold for an extended period of time, shivering thermogenesis is gradually replaced by another mode of heat production not involving muscle contraction, known as nonshivering thermogenesis. A rat that is able to maintain a high metabolic rate in the cold through nonshivering thermogenesis is said to be cold-acclimated.

The major sites of nonshivering thermogenesis are the skeletal muscles and the brown adipose tissue. Although brown adipose tissue is an organ that is especially adapted for producing heat and which proliferates during acclimation to cold, its small size relative to the total body mass of the rat means that its contribution to the overall heat produced in nonshivering thermogenesis compared to the contribution by the skeletal muscles is small. Skeletal muscle is thus the most important site of nonshivering thermogenesis in the cold-acclimated rat.

Nonshivering thermogenesis is controlled by the sympathetic nerve endings. This process can be mimicked by the infusion of noradrenaline into a cold-acclimated rat , which has the ability to increase its metabolic rate four-fold in response to noradrenaline over an animal not acclimated to cold. This large enhancement of the metabolic rate in response to catecholamines is an important characteristic of animals possessing the adaptation for nonshivering thermogenesis.

Although the existence of nonshivering thermogenesis and the involvement of catecholamines in its regulation have been known for several years, the

biochemical mechanism of the process is still not understood. Heat is produced in the cell as a by-product of the oxidation of carbohydrates, lipids and proteins to carbon dioxide and water. The rate of heat production is regulated by the rate of oxidation of substrates by the mitochondrial respiratory chain. Control mechanisms in cellular thermogenic processes involve modification of the rate of mitochondrial respiration. The obvious place in which to look for biochemical differences that might explain nonshivering thermogenesis is thus the mitochondrion. With this in mind studies have been undertaken to characterize in detail muscle mitochondria from cold-acclimated rats since muscle is the major site of heat production in nonshivering thermogenesis and mitochondrial processes regulate heat production within the cell.

There is now evidence that muscle mitochondria undergo alterations associated with the development of nonshivering thermogenesis in the cold-acclimated rat. Morphologically, the mitochondria increase in number but decrease in size, the total mitochondrial mass remaining unchanged. The turnover of certain groups of mitochondrial proteins is increased in the cold-acclimated rat. Oxytetracycline, an inhibitor of mitochondrial protein synthesis, is able to prevent or reverse the observed morphological changes as well as preventing or reversing the cold-acclimated state. Thus, the development and maintenance of nonshivering thermogenesis appears to involve changes in mitochondrial protein synthesis that give rise to mitochondria of altered form and presumably altered function.

Muscle mitochondria in the cold-acclimated rat are also altered biochemically. Increased rates of state 3 (ADP-stimulated) respiration are seen, although ADP/O ratios and respiratory control ratios are normal. The activities of the mitochondrial α-glycerophosphate dehydrogenase and adenine

nucleotide translocase are increased, while the activity of malate dehydrogenase is decreased. The activities of several other mitochondrial and cytoplasmic enzymes are unchanged. Muscle mitochondria in the cold-acclimated rat are thus clearly altered, but the differences observed so far cannot explain the mechanism of nonshivering thermogenesis in this tissue.

Another aspect of mitochondrial function that has yet to be examined in detail in cold-acclimation is the transport of calcium. Mitochondria have the ability to accumulate large amounts of calcium against a concentration gradient in a process linked to the high energy state generated by electron transport. Calcium uptake could thus provide a potential energy dissipating process in mitochondria of the cold-acclimated animal and therefore could be the basis of a method of increasing oxygen consumption and heat production in nonshivering thermogenesis. The maintenance of calcium concentrations in the mitochondrion involves a steady-state between energy-linked uptake and passive release. In other words, calcium is continually recycled across the mitochondrial membrane in a process dependent upon electron transport. Potential mechanisms of increasing heat production in nonshivering thermogenesis would thus include:

(1) An increased rate of energy-linked Ca^{2+} uptake due to an increase in the number of calcium carriers or an increase in their affinity for calcium.

(2) An increased availability of Ca^{2+} for energy-linked uptake due to a noradrenaline-mediated increase in sarcoplasmic calcium levels. This could result from enhanced release of mitochondrial calcium, entry of calcium into the sarcoplasm from the extracellular fluid, or by release of calcium from the sarcoplasmic reticulum.

Of particular importance to this proposed mechanism of nonshivering thermogenesis is the observation that certain physiologically occurring

substances, including cyclic AMP and sodium ion, are capable of inducing release of mitochondrial calcium. The amounts of either of these substances might be increased by noradrenaline action on muscle and thus would provide a means of increasing the availability of calcium for uptake as described above in mechanism (2).

The purpose of the research reported in this thesis is to compare various aspects of calcium transport in muscle mitochondria from cold-acclimated rats and warm-acclimated controls in order to find out whether any differences exist and whether these differences, if found, might give a clue to the mechanism of nonshivering thermogenesis in muscle of the cold-acclimated rat. The problems examined are as follows:

(a) Comparison of the calcium contents of isolated mitochondria.

(b) Comparison of the effects of calcium on mitochondrial respiration.

(c) Comparison of the rates of calcium uptake by muscle mitochondria.

(d) Comparison of the number and affinity of calcium binding sites in muscle mitochondria.

(e) Comparison of the effects of various substances on the release of calcium from muscle mitochondria.

These experiments test the validity of mechanism (1) described above and mechanism (2) in part. The possibility of noradrenaline or other agents increasing sarcoplasmic calcium levels by mechanisms other than release from mitochondria was not studied.

The literature review that follows is divided into three parts. In Part A, the characteristics of cold-acclimation and nonshivering thermogenesis are described as well as the role of the sympathetic nervous system in the process. Part B discusses biochemical mechanisms of heat production in general, with special emphasis being placed on current concepts of non-shivering thermogenesis. The characteristics of mitochondrial calcium

transport and its physiological significance are reviewed in Part C, including evidence for its role in heat production. This review provides a detailed background for the experiments described in the remainder of the thesis.

CHAPTER II: LITERATURE REVIEW

PART A: COLD-ACCLIMATION AND NONSHIVERING THERMOGENESIS

Organisms that can maintain a constant body temperature in spite of fluctuating environmental temperatures are usually referred to as homeotherms. Alternately, animals may be classified according to the source of their body heat (Hochachka & Somero, 1973). Endotherms are those animals that obtain body heat from their own metabolic activities, while ectotherms obtain heat principally from the environment. Thus endotherms include mammals and birds, while most fishes, amphibians and reptiles are ectotherms. The comparative aspects of temperature regulation and the evolution of endothermy is a fascinating study in itself (see Bligh, 1973; Hochachka & Somero, 1973; Stevens, 1973), but will not be discussed here. This review will concentrate on mechanisms of heat production in endotherms, and will use as an example the cold-acclimated rat to show how an endotherm is able to modify its metabolic activities in order to maintain its body temperature under harsh conditions.

1. Cold Exposure and Shivering Thermogenesis

The term "cold exposure" refers to the relatively brief exposure of an animal (up to a few days) to a temperature lower than that to which it is accustomed. For a rat living at usual room temperature (25-28^{o}C) this may range from -20^{o} to 10^{o}C; a commonly used temperature is 4^{o}C. A rat exposed to cold is able to maintain a constant body temperature by bringing into play mechanisms designed to decrease heat loss as well as increase heat production. The sympathetic nervous system, activated by cold stress, gives rise to piloerection and peripheral vasoconstriction which reduce heat loss. The principal mechanism of increasing heat production in the cold-exposed rat is shivering thermogenesis.

Shivering may be defined as a rhythmic, involuntary contraction of the skeletal muscles induced by cold exposure and regulated by the central nervous system (for a review see Hemingway, 1963). It is interesting that although shivering is an autonomic response it takes place in the skeletal muscles, whose contraction is usually under voluntary control. Shivering increases the metabolic rate up to five times the basal level, depending on the species studied and conditions used (Hemingway, 1963). In this respect, the heat produced during the voluntary muscle contractions of light exercise is similar to shivering. As will be discussed in Part B of the review, shivering and exercise produce heat by the same biochemical mechanism.

The sympathetic nervous system plays another important role in cold exposure, namely in the mobilization of the substrates required to support the increased metabolic rate resulting from shivering. Free fatty acids derived from the triglyceride stores of the white adipose tissue are a major fuel; glucose derived from liver glycogen stores and by gluconeogenesis are also used (Himms-Hagen, 1972a). Cold stress stimulates the sympathetic nervous system so that the sympathetic nerve endings and the adrenal medulla secrete noradrenaline and adrenaline respectively; this is indicated by the large increase seen in urinary excretion of these catecholamines and their metabolites (Leduc, 1961; Himms-Hagen, 1975).

2. Cold-Acclimation and Nonshivering Thermogenesis

If a rat remains in the cold for a relatively long period of time, for example, 3-4 weeks at a temperature of 4°, shivering gradually disappears and the rat maintains its body temperature by another mechanism of heat production known as nonshivering thermogenesis. The rat is then said to

be cold-acclimated. This process is illustrated in Fig. 1 (Hart, Héroux & Depocas, 1956). The graph shows that a rat placed in the cold (6°C) will start to shiver immediately, as measured by the increase in muscle electrical activity, but that the intensity of the muscle activity decreases gradually until by day 29 in the cold it is at the same level as the control rats (warm-acclimated) that have lived at 30°C. If the rat is then placed at a lower temperature than the acclimation temperature, shivering will commence again.

In spite of the absence of shivering in the cold-acclimated rat, it is still able to maintain its high metabolic rate by heat-producing mechanisms other than shivering. In fact, if shivering is prevented with the drug tubocurarine, the cold-acclimated rat is still able to maintain a high metabolic rate and thus its normal body temperature in the cold. Curarized warm-acclimated rats, however, become hypothermic and die because they lack the ability to produce heat by nonshivering thermogenesis (Cottle & Carlson, 1956).

Jansky (1973) has defined nonshivering thermogenesis (NST) as "a heat-production mechanism liberating chemical energy due to processes which do not involve muscular contraction." He divides NST into two parts: obligatory or basal NST, which makes up a large part of the basal metabolic rate, and regulatory NST, which refers to extra heat produced in animals exposed to temperatures below the thermoneutral zone necessary to maintain constant body temperature. This definition implies a similar mechanism for heat production during basal metabolism and heat production due to the high metabolic rate in the cold-acclimated animal. Since the biochemical mechanism of NST is still not understood, for the purposes of this thesis nonshivering

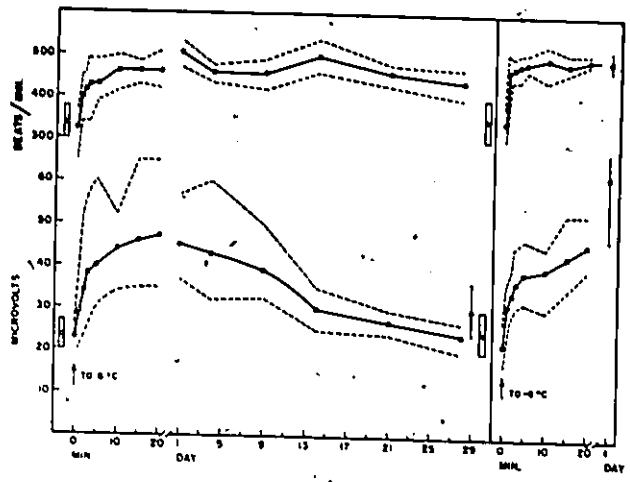

Figure 1. Decrease in Shivering as Measured by Muscle Electrical Activity, During Acclimation to Cold

Muscle electrical activity and heart rate as related to time of exposure to 6°C and -6°C are shown. Solid line in left-hand part of diagram shows muscle electrical activity (lower tracing) and heart rate (upper tracing) in rats living at 6°C. Broken lines show range of variation. Vertical bars at 0 and 29 days show 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, Héroux & Depocas, 1956)

thermogenesis will refer only to the increased heat produced above the basal level in the cold-acclimated animal and due to mechanisms other than muscle movements.

Nonshivering thermogenesis has two key characteristics. First, it is an adaptive process. It occurs only in certain species (eg. rat, rabbit) that have lived in the cold for relatively long periods of time, but is absent from these same species when not cold-acclimated. It also occurs in hibernators (eg. bat, hamster, ground squirrel) but is used only during arousal from hibernation. The newborn of most species (including man) also use NST as a heat producing mechanism, but this ability is lost quite soon after birth and is absent in the adult animal. Second, nonshivering thermogenesis is a facultative process. This means that it can be switched on and off according to the animal's needs. If a cold-acclimated rat is transferred from a cold environment to a warm environment it will turn off NST; if it is then transferred back to the cold it has the ability to turn on and utilize NST once more.

The ability of any particular animal to use nonshivering thermogenesis in place of shivering thermogenesis thus depends on several factors. The process shows species specificity and occurs only in those species having the particular genetic attributes that permit it to hibernate or acclimate to cold. The age of the animal is also important. The newborn of many species possess the capacity for NST but lose it later in life. The adult rat is able to recall the capacity for NST it had at birth by staying in a cold environment for extended periods of time. Old rats, however, show a reduced ability to become cold-acclimated and must rely more on shivering thermogenesis. The relative contributions of shivering and nonshivering

thermogenesis depend also on the time and degree of cold exposure. It was shown in Fig. 1 that shivering decreases as the time of cold exposure increases. A rat fully acclimated to cold at one particular temperature will be forced to rely more on shivering thermogenesis if placed in an even colder environment.

What is the physiological significance of nonshivering thermogenesis? To the newborn animal, even the change from the mother's body temperature of 37°C to a nest temperature of 30°C represents considerable cold stress, and NST plays an important role in maintaining body temperature. Also, certain mammals such as the rat are born immature and are incapable of shivering, and so must rely entirely on NST to prevent hypothermia. Nonshivering thermogenesis is also important for producing the heat required for increasing body temperature to its normal level during arousal of animals from hibernation. The survival of animals in the cold is enhanced by cold-acclimation. For example, a cold-acclimated rat can survive a temperature of -40°C for 200 min while a warm-acclimated rat would die if exposed to only -20°C for the same time period (Jansky, 1973). Even though one may argue that the acclimation to cold of rats in the laboratory is an artificial situation, nonshivering thermogenesis is present in rats kept in outdoor conditions (Héroux, Depocas & Hart, 1959), and so undoubtedly is important as a survival mechanism for animals in the wild. Hart and Jansky (1963) pointed out that heat production resulting from shivering and exercise do not summate but that NST and thermogenesis due to muscular activity are additive. An animal producing heat by NST would thus be able to move about more freely than it would if it were forced to shiver to keep warm.

3. The Role of the Sympathetic Nervous System in Nonshivering Thermogenesis

The sympathetic nervous system is important in the development of nonshivering thermogenesis during cold-acclimation, in the switching on and off of NST, and in the mobilization of substrates required to maintain the high metabolic rate (see reviews by Himms-Hagen, 1967 & 1975). This is brought about principally by the secretion of noradrenaline from sympathetic nerve endings, although catecholamines secreted by the adrenal medulla may also be significant (Himms-Hagen, 1975). Three lines of evidence for the importance of the sympathetic nervous system will be discussed.

(a) Activation of the Sympathetic Nervous System

That the sympathetic nervous system is activated by cold exposure is indicated by the increased urinary excretion of both adrenaline and noradrenaline (Fig. 2, Leduc, 1961). The rate of noradrenaline excretion increases about five times upon cold exposure and remains at a high level during and after the acclimation period. Adrenaline levels, on the other hand, increase during the first week of cold exposure and decline shortly thereafter, returning to the control values by the time full acclimation to cold is achieved. Shum, Johnson and Flattery (1969) found increased excretion of the major metabolites of noradrenaline, normetanephrine and 3-methoxy-4-hydroxy-phenylglycol (MHPG), during cold acclimation. Adrenaline metabolites metanephrine and 3-methoxy-4-hydroxymandelic acid (MHMA) also increase. This implies an increased rate of synthesis of adrenaline in the adrenal medulla and of noradrenaline in the sympathetic nerve endings. In the case of noradrenaline, this increased synthesis remains at a high level even after acclimation to cold (see review by Himms-Hagen, 1975).

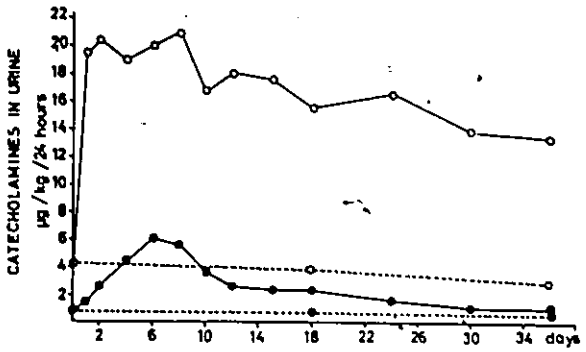

Figure 2. Urinary Excretion of Adrenaline and Noradrenaline During Cold-Acclimation

The graph shows the urinary excretion of adrenaline (●) and noradrenaline (o) in rats weighing 170-180 g at 3°C (solid line) and 22°C (dashed line). Each point represents the mean of 6 individual rats.

(From Leduc, 1961)

(b) The Mimicking Effects of Catecholamines

Hsieh and Carlson (1957) demonstrated that noradrenaline administered by intramuscular injection produces a much greater calorigenic response in cold-acclimated rats compared to warm-acclimated rats. Fig. 3 (Himms-Hagen, 1970) illustrates the difference in oxygen consumption rates between a warm-acclimated rat and a cold-acclimated rat during the intravenous infusion of noradrenaline. Typically, a cold-acclimated rat shows a four-fold enhancement of oxygen uptake compared to controls. Fig. 3 also shows that before infusion, the warm-acclimated and cold-acclimated rats have similar basal metabolic rates, that is, nonshivering thermogenesis is switched off in the cold-acclimated rat in the warm environment in which the experiment is performed. However, infusion of noradrenaline produces a rapid increase in oxygen uptake corresponding to the switching on of nonshivering thermogenesis. At the end of the infusion period the metabolic rate declines to the basal level as NST is switched off again.

Although Hsieh and Carlson (1957) found a much smaller effect of adrenaline on oxygen uptake in the cold-acclimated rat compared to noradrenaline, Himms-Hagen (1969) showed that by infusing adrenaline intravenously rather than administering it by intramuscular injection, an enhancement of metabolic rate equal to that induced by noradrenaline can be achieved (Fig. 4).

The development of the enhanced response to catecholamines in the cold-acclimated rat is closely associated with the replacement of shivering thermogenesis with nonshivering thermogenesis (Fig. 5, Depocas, 1960). Comparing Fig. 1 with Fig. 5, it is clear that the time course of the decline of shivering and the increase in the enhanced response to noradrenaline is the same. Further evidence that noradrenaline-induced thermogenesis is related to nonshivering thermogenesis is the observation that curarized

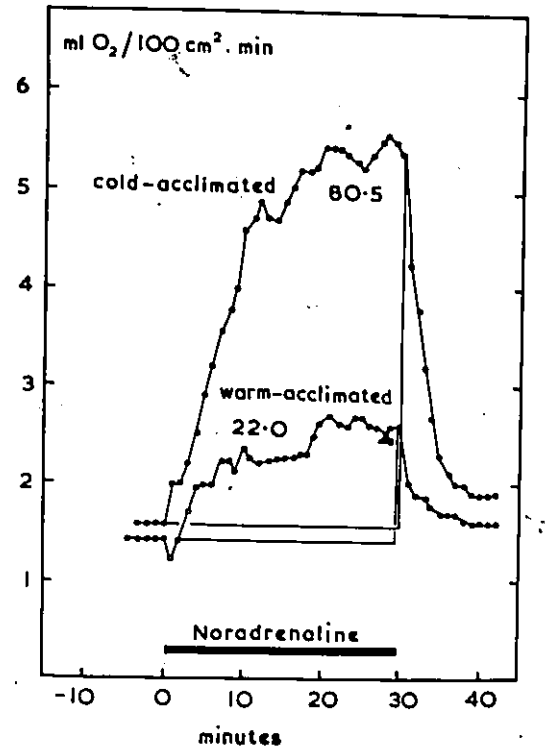

Figure 3. Enhancement of Calorigenic Response to Noradrenaline by Cold-Acclimation

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 per min) from 0 to 30 min. Rats were lightly anesthetized with sodium pentobarbital. Warm-acclimated rat weighed 478 g, and cold-acclimated rat weighed 373 g; they had lived at room temperature (25-28^{o}C) and in the cold (4^{o}C), respectively, for 13 weeks, and their weights at the start of the acclimation period were 202 g and 192 g respectively. Values of 80.5 and 22.0 on the graph are obtained from the area under the curve during the 30 min of infusion of noradrenaline, and they represent total increase in oxygen uptake in ml O_2/100 cm^2/30 min. Increases shown are typical of rats kept under these conditions.

(From Himms-Hagen, 1970)

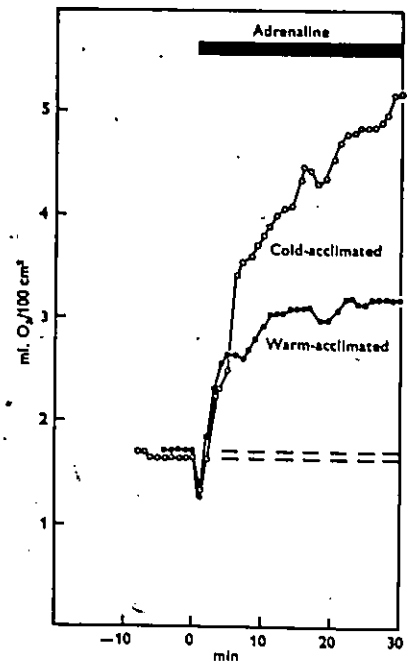

Figure 4. Enhancement of the Calorigenic Response to Adrenaline by Cold-Acclimation

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 per min) from 0 to 30 min. Rats were lightly anesthetized with sodium pentobarbital. Warm-acclimated rat weighed 415 g; cold-acclimated rat weighed 331 g; they had lived at room temperature (25-28^{o}C) and 4^{o}C respectively for 9 weeks.

(From Himms-Hagen, 1969)

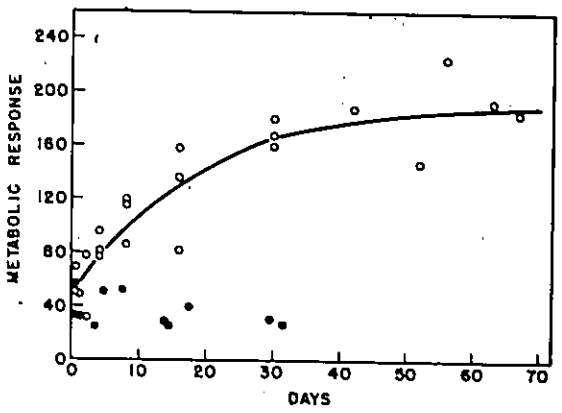

Figure 5. Development of Enhanced Calorigenic Response to Noradrenaline During Acclimation to Cold

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

(From Depocas, 1960)

cold-acclimated rats still show the enhanced response to noradrenaline even though muscle movements cannot take place (Cottle and Carlson, 1956).

If a cold-acclimated rat is removed from the cold and placed in a warm environment it will undergo a gradual deacclimation to cold, characterized by the loss of the ability to produce heat by nonshivering thermogenesis. This is accompanied by the gradual disappearance of the enhanced metabolic response to noradrenaline (Bartunkova, Jansky & Mejsnar, 1971).

That noradrenaline is involved directly in the development of adaptation to cold is indicated by experiments of LeBlanc and Pouliot (1964). Rats given prolonged treatment with noradrenaline developed an adaptive state similar to that of cold-acclimation in that they showed increased cold resistance and an enhanced calorigenic response to noradrenaline.

The enhanced metabolic response to noradrenaline thus mimics closely the adaptive state of the animal with respect to its degree of cold-acclimation as well as mimicking the facultative process of switching on and off of NST. For this reason measurement of the calorigenic action of noradrenaline is very useful experimentally as an indicator of nonshivering thermogenesis.

(c) Inhibition of the Sympathetic Nervous System

A variety of surgical, immunological and pharmacological techniques have been used in attempts to inhibit the functioning of the sympathetic nervous system in order to assess its role in the development and regulation of nonshivering thermogenesis in the cold-acclimated animal. These include adrenodemedullation or adrenalectomy, immunosympathectomy, the use of drugs such as reserpine, ganglionic blocking agents (eg. hexamethonium, chlorisondamine), adrenergic blocking agents (eg. guanethidine), and inhibitors of catecholamine synthesis. The effects of these agents in relation to cold-

acclimation have been reviewed recently by Himms-Hagen (1975) and so will not be described to any extent here.

Although the use of these techniques support the direct role of the sympathetic nervous system in nonshivering thermogenesis, problems of interpretation arise because it is often impossible to inhibit completely all parts of the sympathetic nervous system. The rat is sufficiently adaptable so that it can make use of regulatory mechanisms other than those directly inhibited by the particular technique employed. The relative roles of the two divisions of the sympathetic nervous system (the adrenal medulla and the sympathetic nerve endings) in cold-acclimation are still not clear (Himms-Hagen, 1975).

4. Sites of Nonshivering Thermogenesis

The identity of the tissue or organ which contributes most to the increased heat production in nonshivering thermogenesis has long been controversial. Early workers observed increases in the temperature of the liver and other internal organs upon exposure of animals to cold, but such experiments yield little useful information of a quantitative nature since they do not take into account the blood flow to the particular organ. The best method of estimating the thermogenesis of an organ is to measure the blood flow through the organ and the removal of oxygen from the blood (Jansky, 1973). However, the complicated vascularization of some tissues and the minute diameter of blood vessels in small animals such as the rat make such procedures technically difficult, and often indirect methods must be used.

Depocas (1958, 1960) was able to functionally eviscerate rats by restricting blood flow through the liver, intestines, stomach, spleen and pancreas. He demonstrated that cold-acclimated, functionally eviscerated rats

still showed an enhanced metabolic response either to cold exposure or infused noradrenaline, thus proving that the liver and other viscera are not important for nonshivering thermogenesis. The major sites of NST must thus be extravisceral, the skeletal muscle being a good candidate because of its large mass.

Jansky and Hart (1963) looked directly at the oxygen uptake of the hind-limb muscles of curarized, cold-acclimated rats by measuring arterio-venous oxygen contents and blood flow values, and found that either cold exposure or noradrenaline administration doubled the rate of oxygen consumption compared to controls. Although this experiment affirms the importance of skeletal muscle as a site of nonshivering thermogenesis, it gives no indication of the relative contribution of the whole muscle mass to the total heat produced in NST. To solve this problem, Jansky (1961, 1963) proposed an indirect method based on the assumption that total cytochrome oxidase activity is an indicator of the maximum metabolic capacity of either the whole animal or of various organs measured individually. Using such methods, it was estimated that 50-70% of NST originates in the skeletal muscles (Jansky, 1963, 1966). Jansky and Hart (1968) measured total muscle blood flow in cold-acclimated rats exposed to cold and calculated that if all the oxygen flowing to the muscles were consumed the muscles would contribute no more than 50% to NST. It must be kept in mind that these indirect methods do not indicate the true amount of oxygen consumed by the muscle mass and so the contribution of muscle to NST estimated by Jansky may be incorrect. However, experiments of this type emphasize that muscle is an important site of NST, and similar experiments are useful in ruling out other tissues as sites of NST.

The brown adipose tissue has long been regarded as an important site of nonshivering thermogenesis because it is particularily well adapted for the production of heat. It has a rich sympathetic innervation and blood supply, and is packed with mitochondria and multilocular lipid droplets. The mitochondria have a high respiratory capacity as indicated by their abundance of tightly packed, regularily arranged cristae. Under sympathetic stimulation the lipid droplets supply large quantities of free fatty acids used as fuel by the mitochondria to support the resulting large increase in respiration rate (see review by Smith & Horwitz, 1969). In spite of these features, several lines of evidence argue against a role for the brown adipose tissue as a major locus of nonshivering thermogenesis in the cold-acclimated rat. Removal of the interscapular brown adipose tissue, which comprises about one-third of the total, causes little immediate decrease in the enhanced calorigenic response to noradrenaline (Himms-Hagen, 1969). The extra oxygen must thus be consumed by other tissues. Calculations based on blood flow suggest a maximum possible contribution by brown adipose tissue of 6-8% of the total oxygen consumption (Jansky & Hart, 1968; Imai, Horwitz & Smith, 1968). However, in the newborn rabbit, where the brown adipose tissue comprises a greater percentage of the total body weight compared to the rat (5% versus less than 1%), up to 80% of the total heat produced during noradrenaline infusion can be attributed to the brown adipose tissue (Hull & Segall, 1965).

Even though it appears that the brown adipose tissue does not contribute significantly to NST in the cold-acclimated rat, it may have another important function that will be discussed in the next section.

Table 1 summarizes the relative contributions of the various organs to nonshivering thermogenesis.

TABLE I

Relative Contributions of Various Organs to Nonshivering Thermogenesis

Organ	Relative Contribution to NST (%)	Method of Estimation	Reference
Skeletal Muscle	50-70%	cytochrome oxidase	Jansky, 1963, 1966
	50%	blood flow	Jansky & Hart, 1968
Brown Adipose Tissue	6%	blood flow, O_2 uptake	Jansky & Hart, 1968
	8.2%	heat exchange	Imai et al., 1968
	12%	removal	Himms-Hagen, 1969
Liver	up to 25%	blood flow	Jansky & Hart, 1968
Intestines	<10%	blood flow, O_2 content	Jansky, 1971
Kidneys	5%	cytochrome oxidase	Jansky, 1966
	0	blood flow, O_2 content	Jansky & Hart, 1963
Skin	<6%	cytochrome oxidase	Jansky, 1966

5. Special Role of Brown Adipose Tissue in Nonshivering Thermogenesis

Although the contribution of brown adipose tissue to overall heat production in nonshivering thermogenesis does not appear to be great, it may be important in the supression of shivering and in the promotion and maintenance of NST (Smith & Horwitz, 1969; Himms-Hagen 1969, 1970, 1972a)

Even though brown adipose tissue comprises only about 1% of the total body weight of the rat, it is strategically located around major blood vessels and nerve tracts in the thoracic and cervical regions; deposits also extend along the spinal cord to the renal area. Brown fat thus functions as a localized "internal heating jacket" that protects important blood vessels and nerves from cold stress (Smith and Horwitz, 1969). The interscapular brown adipose tissue (IBAT), which makes up one-third of the total brown fat, is located near spinal cord thermoreceptors that are responsible for the control of shivering (Brück & Wünnenberg, 1966). Thus, Smith and Horwitz (1969) pointed out that venous drainage of the IBAT via Sulzer's vein would result in the transfer of heat to these thermoreceptors thereby inhibiting shivering. Indeed, hyperplasia and increased vascularization of the IBAT during cold-acclimation is accompanied by a decrease in shivering concomitant to the increase in nonshivering heat production.

In addition to this thermogenic role of brown adipose tissue, Himms-Hagen (1969) has proposed an endocrine function for this tissue. More specifically, she postulated that brown fat secretes a factor responsible for the development and maintenance of NST in other tissues, principally skeletal muscle. This hypothesis was based on the observation that removal of the IBAT from cold-acclimated rats resulted in little immediate reduction in the enhanced calorigenic response to noradrenaline, but four days after the

operation the increased response was reduced by 40% compared to sham-operated controls (Fig. 6, Himms-Hagen, 1970). Similar reductions have been observed by several other workers (Leduc & Rivest, 1969; Hayward & Davies, 1972; Laury & Portet, 1974), although Flattery & Sellers (1972) and Foster (1974) did not find any difference between controls and rats lacking the IBAT. The reduction observed by Himms-Hagen and others could not be accounted for by the absence of potential oxygen consumption by the IBAT because of the small size of the tissue removed and the lack of reduction in oxygen consumption shortly after the operation. Replacement of pieces of the removed IBAT back into the peritoneal cavity considerably reduced the loss of the calorigenic response to noradrenaline (Fig. 6). Presumably, this is because the postulated factor is still able to enter the bloodstream. However, injections of homogenates or other preparations of IBAT are ineffective and so far no success has been achieved in the isolation and identification of the proposed factor.

A different approach to the study of the role of brown adipose tissue in NST was introduced by Himms-Hagen (1971). Because the growth of brown adipose tissue during cold-acclimation is accompanied by a rapid proliferation of mitochondria, the inhibitor of mitochondrial protein synthesis oxytetracycline was injected into rats acclimating to cold in an attempt to prevent mitochondrial changes from taking place. Treatment of rats living at $4^{\circ}C$ for 2 weeks did not prevent growth of the whole tissue but it effectively prevented mitochondrial proliferation as measured by cytochrome oxidase activity. Most significantly, the cold-induced increase in the enhanced calorigenic response to noradrenaline was also inhibited (Fig. 7, Himms-Hagen 1971). This suggests that mitochondrial protein synthesis in brown adipose tissue plays a role in the promotion of the development by

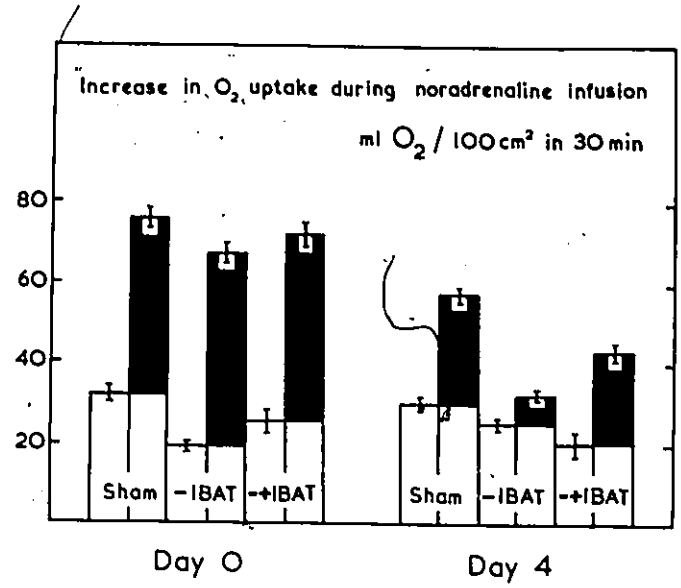

Figure 6. Effect of Removal and Replacement of Interscapular Brown Adipose Tissue of Warm-Acclimated and Cold-Acclimated Rats on the Calorigenic Response to Noradrenaline

Values shown represent the total increase in oxygen uptake during a 30-min infusion of noradrenaline. Each bar is the mean of 4 experiments ± S.E.M. Cold-acclimated rats are represented by bars with black upper portions and warm-acclimated controls are represented by open bars to the left. The blackened portion of a bar is the difference between the response of cold-acclimated rats and the response of the corresponding control warm-acclimated rats; it is a measure of 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, anesthetized with ether, and operated on as follows: (a) sham-operated (Sham on graph); (b) interscapular brown adipose tissue removed (-IBAT); and (c) interscapular brown adipose tissue removed, cleaned and weighed, cut into 4 or 5 portions, and replaced in the peritoneal cavity (-+IBAT); a similar laparotomy was performed also on rats in groups (a) and (b). Rats remained at room temperature until infusion of noradrenaline. (From Himms-Hagen, 1970)

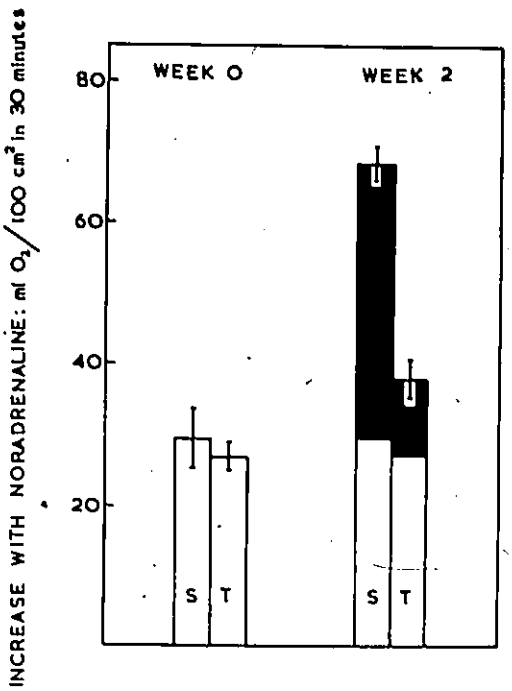

Figure 7. Effect of Oxytetracycline Administration During Acclimation to Cold on the Enhanced Calorigenic Response to Noradrenaline

The black portion of the bars represents the enhancement of the calorigenic response to noradrenaline due to the 2 weeks of cold-acclimation. Oxytetracycline (Terramycin, Pfizer, 250 mg in 3 ml) was administered intramuscularly, twice daily, for 2 weeks; the total dose was 200 mg/kg per day (T). Control rats received i.m. injections of saline (S).

(From Himms-Hagen, 1971)

other tissues of the capacity to respond calorigenically to noradrenaline. This point will be discussed further in Part B of the review.

Figure 8 (Himms-Hagen, 1972a), summarizes the events taking place during cold-acclimation. Cold exposure activates the sympathetic nervous system which brings into play mechanisms designed to reduce heat loss (peripheral vasoconstriction, piloerection). Increased heat production is brought about at first by shivering thermogenesis, regulated by the central nervous system. During acclimation, shivering declines and heat is produced by nonshivering thermogenesis controlled by the sympathetic nervous system. The major site of nonshivering thermogenesis is the skeletal muscles. Brown adipose tissue is important in the supression of shivering by the warming of spinal thermoreceptors and in the promotion of nonshivering thermogenesis, possibly by the secretion of a factor that influences other tissues to respond calorigenically to noradrenaline. Throughout, the sympathetic nervous system in response to cold mobilizes the substrates necessary to maintain the high metabolic rate required to support both shivering and nonshivering thermogenesis.

PART B: BIOCHEMICAL MECHANISMS OF HEAT PRODUCTION

The purpose of this section is to review the biochemical aspects of intracellular heat production. This will be done by first considering cellular thermogenesis in general and then looking in more detail at current concepts of the biochemical mechanisms of nonshivering thermogenesis.

1. Theories of Cellular Thermogenesis

Heat is produced in the cell as a by-product of the oxidation of the carbohydrates, lipids and proteins derived from ingested foodstuffs, and

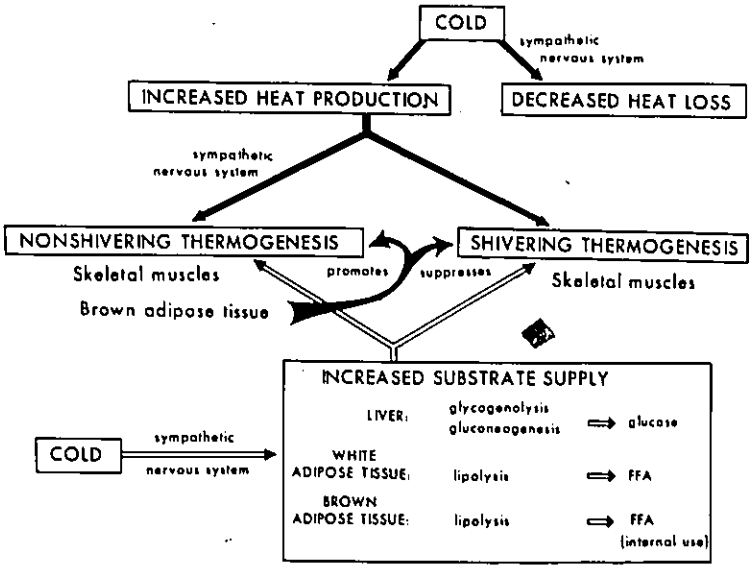

Figure 8. Summary of Events Taking Place During Cold-Acclimation

Refer to the text for a description.

(From Himms-Hagen, 1972a)

endotherms have evolved a number of control mechanisms designed to regulate heat-producing metabolic reactions. Using thermodynamic arguments, Prusiner and Poe (1968, 1970) showed that the major site of heat production in the cell is the mitochondrion and that the major heat producing process consists of electron transport and oxidative phosphorylation. If one considers the total free energy change during the complete combustion of glucose, fatty acids and amino acids to carbon dioxide and water, virtually all of the free energy results from the oxidation of the reduced pyridine nucleotides ultimately derived from these precursors. Since oxidative phosphorylation is only about 25% efficient (Prusiner & Poe, 1968), then 75% of this large free energy change is dissipated as heat. It follows that any process leading to an increased rate of respiration will be a thermogenic process, and theories of cellular thermogenesis usually involve mechanisms by which respiration rate is increased. Recent studies by Wilson and coworkers (eg. Wilson et al., 1974) have demonstrated that the reactions of the electron transport chain are in near equilibrium with the phosphorylation of ADP. This means that the free energy change associated with oxidative phosphorylation is zero and the efficiency of the process approaches 100% in tightly coupled mitochondria rather than 25% as estimated earlier by Prusiner and Poe. The rate of heat production would still be dependent on mitochondrial respiration rate, but the reactions responsible for heat production would include various other reactions in the cell rather than just those of electron transport and oxidative phosphorylation.

Control of cellular thermogenic processes can be accommodated by two major theories. The first theory requires that the mitochondrion be fully coupled in the classical sense, in other words, respiration rate is

dependent on the level of phosphate acceptor (ADP) in the cell. More recently, the concept of the phosphorylation state ratio, {ATP}/{ADP}{Pi} as the regulator of respiration rate has been introduced (Wilson et al., 1973; Owen & Wilson, 1974). Any process that decreases the phosphorylation state ratio, that is, by utilizing ATP and increasing the intracellular concentration of ADP and Pi, will increase respiration rate and thus heat production. The assumption is made that the substrate and oxygen supply is not rate-limiting. Synthetic pathways that utilize large amounts of ATP have often been implicated in heat production, especially if coupled with the corresponding catabolic pathway in order to provide a futile cycle or net ATPase. An example of this is the synthesis of triglycerides from fatty acids and glycerol followed by the breakdown of the triglyceride back to fatty acids and glycerol (eg. see Himms-Hagen, 1965; Prusiner & Poe, 1970). Specific ATPases have also been shown to be involved in thermogenesis, the best example being the myosin ATPase of muscle contraction that gives rise to the increased respiration rates seen in shivering and exercise. The Na^+K^+-ATPase of the plasma membrane, which pumps Na^+ out and K^+ into the cell and which maintains the transmembrane potential difference, is another example. Several workers consider the Na^+K^+-ATPase to be the pacemaker of cellular respiration and responsible for heat production seen during basal metabolism, hyperthyroidism and cold-acclimation (Whittam, 1961; Ismail-Beigi & Edelman, 1970; Edelman, 1974; Stevens, 1973; Stevens & Kido, 1974; Horwitz & Smith, 1972). Other ion-pumping ATPases, such as the Ca^{2+}-ATPases of the sarcoplasmic reticulum and plasma membrane, conceivably could be involved in thermogenesis also.

The second major theory of cellular thermogenesis is basically different from the first in that the mitochondria undergo loss of respiratory control

and become either uncoupled or loosely coupled. The rate of respiration is then no longer sensitive to the phosphorylation state ratio but is regulated by substrate availability and by the presence of specific uncoupling or loose coupling agents. Uncoupled mitochondria respire at maximal rates and do not phosphorylate ADP; the action of 2, 4-dinitrophenol (DNP) produces this effect in vitro. Loosely coupled mitochondria also show loss of respiratory control with ADP but are still capable of carrying out oxidative phosphorylation. Uncoupling effects have at various times in the past been used to explain hyperthyroidism (Hoch, 1962) and nonshivering thermogenesis in liver (Hannon, 1959) and brown adipose tissue (Prusiner & Poe, 1968, 1970). These hypotheses now appear to be incorrect as will be discussed later. There is quite good evidence, however, for a mechanism of NST in brown adipose tissue involving loosely coupled mitochondria (see review by Flatmark & Pedersen, 1975).

A special case of loose coupling is referred to by Prusiner and Poe (1968) as "oligomycin-insensitive respiration (but sensitive to uncouplers)." Oligomycin inhibits coupled respiration by blocking oxidative phosphorylation at the step where ADP is phosphorylated. Uncouplers such as DNP can release the inhibitory effects of oligomycin on respiration but not on ATP synthesis. The energy-linked accumulation of Ca^{2+} and K^{+} by mitochondria are two processes that are insensitive to oligomycin but sensitive to DNP, and which represent alternate pathways of utilizing the high energy state generated by electron transport (Lehninger, Carafoli & Rossi, 1967). This would be equivalent to the loosening of coupling of respiration from oxidative phosphorylation. Transport of Ca^{2+} by mitochondria is a well-established phenomenon and its characteristics and possible role in thermogenesis will

be described in detail in Part C. Uptake of K^+ by mitochondria *in vitro* requires the presence of an ionophorous antibiotic such as valinomycin, although free fatty acids have been suggested as natural K^+ ionophores in brown adipose tissue mitochondria and a fatty acid-induced energy dissipating cyclic K^+ flux has been proposed as a thermogenic mechanism in that tissue (Reed & Fain, 1968).

In summary, heat is produced in the mitochondrion as a result of respiration. The rate of heat production is dependent on the overall respiration rate which is regulated either by substrate availability, the phosphorylation state ratio, the presence of agents causing loose coupling, and/or the availability of ions such as Ca^{2+} for energy-linked uptake.

2. Some Examples of Thermogenic Processes

In a recent review, Himms-Hagen (1976) has discussed the mechanisms of heat production in a variety of physiological and pathological conditions. For this reason, the examples to be given in this section will not be dealt with in any great detail but serve only to illustrate the theories described in the previous section.

(a) Basal Metabolic Rate

The basal level of thermogenesis is no doubt due to ATP and substrate utilization by a variety of synthetic, mechanical and osmotic processes required to keep the body alive. Regulation is thus largely achieved via the phosphorylation state ratio. The Na^+K^+-ATPase has been proposed as a pacemaker of respiration and a large part of the basal metabolic rate has been attributed to it (Whittam, 1961). The activity of this enzyme has been shown to be correlated with the thyroid status of rats (see review by Edelman, 1974), and thyroid hormones are known to regulate the basal metabolic rate. Evidence for the Edelman hypothesis is based largely on experiments in which ouabain or lack of Na^+ is used to inhibit the Na^+K^+-ATPase

and the resulting decrease in respiration rate is considered to be a measure of that portion of the metabolic rate due to the functioning of this enzyme. However, Himms-Hagen (1976) has criticized the use of ouabain in obtaining such measurements because it causes changes in ion composition, particularily K^+ depletion and an altered Ca^{2+} distribution, which could affect cellular respiration in itself. Thus the quantitative importance of the Na^+K^+-ATPase in regulating the basal metabolic rate is not clear.

(b) Hyperthyroidism

This condition is characterized by an elevated basal metabolic rate. Earlier studies showed that liver and skeletal muscle mitochondria undergo increases in size and respiratory capacity as a result of thyroid hormone treatment (Tata, 1964; Gustafsson et al., 1965). More recent studies, however, have indicated that the changes in skeletal muscle mitochondria take place only after prolonged thyroid hormone treatment (Winder et al., 1975), and so are probably associated with thyrotoxicosis and not mild hyperthyroidism.

An early explanation for the increased heat production seen in hyperthyroidism was that thyroid hormones caused uncoupling of mitochondria (see review by Hoch, 1962). Indeed, liver mitochondria isolated from thyroid hormone-treated animals showed increased state 4 respiration rates and reduced P/O ratios. This theory fell into disfavor when tightly-coupled mitochondria from liver and skeletal muscle were isolated from hyperthyroid animals by a number of workers. Currently, an increased activity of Na^+K^+-ATPase is thought to be largely responsible for the elevated basal metabolic rate seen in hyperthyroidism. Ouabain or lack of Na^+ largely inhibits the increased oxygen uptake of liver, kidney and muscle slices from hyperthyroid

rats (Ismail-Beigi & Edelman, 1970, 1971). The activity of the Na^+K^+-ATPase in liver, kidney and muscle is also higher in hyperthyroid animals (Ismail-Beigi & Edelman, 1971; Asano, Liberman & Edelman, 1976). Present evidence favors a stimulation of the Na^+K^+-ATPase due to the synthesis of more Na^+ pump sites or to the unmasking of pump sites already present. This comes about through the action of a protein induced by thyroid hormone at the transcriptional level (Edelman, 1974).

(c) Muscle Activity

Muscle activity, both shivering and exercise, results in heat production through changes in the phosphorylation state ratio, since muscle contraction involves the use of large amounts of ATP by the myosin ATPase. Ion pumping by the Na^+K^+ATPase during repolarization and by the sarcoplasmic reticulum Ca^{2+}-ATPase during relaxation would also contribute. As mitochondrial Ca^{2+}-accumulation has been implicated in relaxation of heart and red skeletal muscle (Lehninger, 1974a), this energy-dissipating process might contribute further to the total heat produced.

(d) Malignant Hyperthermia

This is an interesting pathological condition in which susceptible individuals are sensitive to certain inhaled anesthetics such as halothane, responding with extreme muscle rigidity and hyperthermia (see Gordon, Britt & Kalow, 1973). Apparently, the sarcoplasmic reticulum Ca^{2+}-uptake mechanism in these patients becomes inhibited by halothane and so Ca^{2+} accumulates in the cytosol. This accounts for the failure of the muscle to relax. The mitochondria respond to elevated cytosolic Ca^{2+} levels by accumulating the Ca^{2+} - this in itself would produce heat - until eventually such excessive amounts are taken up that the mitochondria become damaged and uncoupled.

Respiratory rates would then occur at very high levels.

(e) Luft Hypermetabolic Syndrome

This is a very rare condition characterized by an elevated basal metabolic rate of non-thyroid origin (Luft et al., 1962). The muscle mitochondria are present in increased number and many are morphologically bizarre. Respiration rates are very high and there is no respiratory control with ADP. The P/O ratios are normal or slightly lowered suggesting that the mitochondria are loosely coupled. Chloramphenicol, known to inhibit mitochondrial protein sysnthesis, has been used to reduce the elevated basal metabolic rate by inhibiting the rapid proliferation of mitochondria in this disease (Abu Haydar et al., 1971; Afifi et al., 1972).

3. Nonshivering Thermogenesis - Biochemical Mechanisms

In Part A the characteristics and significance of nonshivering thermogenesis were described, largely from a physiological point of view: Even though the existence of NST and the involvement of the sympathetic nervous system in the process has been established for quite a while, an exact description of events at the molecular level has proved elusive. Research into the biochemical mechanism of NST should yield interesting information about cellular thermogenic processes, in particular about their regulation by the neuroendocrine system.

(a) Nonshivering Thermogenesis in Brown Adipose Tissue

Much attention has been focussed on BAT because of its special heat-producing function, and now detailed biochemical mechanisms of nonshivering thermogenesis have been proposed for this tissue.

BAT mitochondria play a central role in heat production in BAT because they have unique properties geared especially to thermogenesis (Flatmark

& Pedersen, 1975). Compared to other mitochondria, BAT mitochondria have a greater respiratory capacity reflected in higher levels of cytochromes and respiratory enzymes. In thermogenic states, such as cold-acclimation, the mitochondrial mass increases as does the respiratory capacity. Noradrenaline-induced lipolysis produces large amounts of fatty acids and glycerol that are the principal fuel for supporting the high respiration rate in the mitochondria. The mitochondria are particularily suited for this as they show a high capacity for the oxidation of free fatty acids and α-glycero-phosphate. Free fatty acids (or their CoA esters) are thought to have another important function, namely in the reversible loosening of coupling of electron transport from oxidative phosphorylation.

When isolated by conventional techniques, BAT mitochondria are uncoupled regardless of whether or not the tissue is in a thermogenic state. This is due to the relatively large amount of free fatty acids that become bound to the mitochondria during isolation. By careful manipulation of incubation conditions it is possible to recouple the mitochondria to varying degrees. Either bovine serum albumin (eg. Grav, Pedersen & Christiansen, 1970), or carnitine plus ATP (Hittleman, Lindberg & Cannon, 1969) will effect recoupling, probably as a result of the removal of the uncoupling fatty acids. Significantly, the degree of recoupling achieved by these treatments is related to the thermogenic state of the animal. For example, BAT mitochondria from newborn or cold-exposed guinea-pigs show reduced P/O ratios compared with warm-adapted or warm-readapted guinea-pigs (Christiansen, Pedersen & Grav, 1969). Tight coupling in the mitochondria from thermogenic BAT can be achieved by adding a nucleoside di- or triphosphate (eg. ADP, ATP, GDP, GTP),

and a regulatory role for these compounds has been suggested (Skaane et al., 1972). Possibly they act by inducing a membrane conformational change thereby negating the uncoupling effect of the fatty acid. The main point is that thermogenic BAT mitochondria undergo an adaptive change so that the coupling state becomes sensitive to the presence of purine nucleotides.

The fundamental difference seen in BAT mitochondria is an unusual sensitivity to fatty acids resulting in a leakage of the so-called primarily conserved energy (Flatmark & Pedersen, 1975), which is the high energy state generated by electron transport used to drive oxidative phosphorylation and cation transport and to generate the transmembrane electrochemical potential gradient. BAT mitochondria are considered to be loosely coupled rather than uncoupled because even though there is no respiratory control in the absence of albumin, oxidative phosphorylation can still be detected (Grav et al., 1970), as can the accumulation of Ca^{2+} (Hittleman, Fairhurst & Smith, 1967).

In summary, nonshivering thermogenesis in BAT is mediated by noradrenaline through activation of the plasma membrane adenylate cyclase to produce cyclic AMP, which results in the enhancement of lipolysis of the triglyceride stores to produce free fatty acids. The free fatty acids serve a dual role, first as substrates for the enhanced mitochondrial respiration, and secondly in the loosening of coupling such that the rate of respiration becomes independent of the phosphorylation state ratio and therefore occurs at a high rate. When the noradrenaline stimulus is removed, cyclic AMP levels decline and lipolysis is slowed. Fatty acids are then removed and recoupling occurs under the influence of purine nucleotides.

An alternate theory of nonshivering thermogenesis in BAT analogous to the Edelman hypothesis for hyperthyroidism has been proposed by Horwitz, namely that increased operation of the Na^+K^+-ATPase is responsible for a large part of the noradrenaline-induced increase in oxygen consumption seen in BAT during cold stress. Either electrical stimulation of the nerves leading to BAT or administration of noradrenaline depolarizes the plasma membrane (Horwitz, Horowitz & Smith, 1969; Girardier, Seydoux & Clausen, 1968), resulting from an increased permeability of the membrane to Na^+ (Horowitz, Horwitz & Smith, 1971; Girardier & Seydoux, 1971). This ionic imbalance results in a stimulation of the Na^+K^+-ATPase. Noradrenaline has also been shown to have a direct stimulatory effect on the Na^+K^+-ATPase (Herd, Horwitz & Smith, 1970). Ouabain or lack of Na^+ reduces the calorigenic effect of noradrenaline on isolated hamster brown fat cells by 60% (Horwitz, 1973). The increased utilization of ATP by the Na^+K^+-ATPase is thus thought to increase mitochondrial respiration via a change in the phosphorylation state ratio. The free fatty acids resulting from noradrenaline-induced lipolysis would serve only as respiratory substrates in the Horwitz proposal, not as uncoupling agents. This theory is therefore incompatible with the loose coupling hypothesis described previously because it requires the mitochondria to be in the coupled state. Himms-Hagen (1976) suggested one way of incorporating the observations of both theories. Possibly loose coupling does indeed occur and respiration rate becomes dependent on substrate supply. This would require increased activity of glycolysis and the tricarboxylic acid cycle. An increased formation of ADP by the Na^+K^+-ATPase would be required to provide the ADP necessary for substrate level phosphorylation reactions in these pathways.

(b) Nonshivering Thermogenesis in Skeletal Muscle

Relatively little is known about nonshivering thermogenesis in muscle at the biochemical level. In searching for adaptive changes that might take place in muscle in association with cold-acclimation, it is useful to consider three different sites where the changes might manifest themselves (Himms-Hagen, 1976): 1. the noradrenaline receptor system (presumed to involve adenylate cyclase), 2. the link between the receptor system and the final metabolic response (presumed to involve cyclic AMP) and 3. the final metabolic response itself (presumed to occur in the mitochondria).

No change is found in the adenylate cyclase system of muscle (basal activity, catecholamine-stimulated activity, or fluoride-stimulated activity) of cold-acclimated rats, although increases in catecholamine and fluoride-stimulated activity are seen during the first week of cold exposure (Muirhead & Himms-Hagen, 1974). This transient increase is thought to be associated with shivering because it disappears by the time the rat is fully acclimated to cold. Cold-acclimated rats also excrete more cyclic AMP in their urine but no more than warm-acclimated rats exposed to cold for the first time and using shivering thermogenesis (Muirhead, Inglis & Himms-Hagen, 1974). It appears then that no change takes place in the receptor system for noradrenaline as a result of cold-acclimation.

The nature of the link between the receptor system and the mitochondrion is not known, but it is logical to assume that it involves cyclic AMP as well as a protein kinase that could influence the mitochondria in some manner either directly or indirectly. Noradrenaline is also known to influence ion movements in muscle and it is conceivable that changes in the ionic environment could play a role in noradrenaline-induced thermogenesis. At

present this is a largely unexplored area, but it may yield interesting results in future research.

The logical place to look for adaptive changes in the cold-acclimated rat is in the muscle mitochondria, since the mitochondrion is the site of oxygen consumption and alterations are known to take place in muscle mitochondria but they are of a quite different nature than those found in brown adipose tissue. Muscle mitochondria become smaller in size and more numerous, with the total mitochondrial mass as measured by cytochrome oxidase activity remaining unchanged (Fig. 9, Himms-Hagen et al., 1975; Behrens & Himms-Hagen, 1976). In BAT the mitochondria also increase in number but they increase in size and become more densely packed with cristae, the net result being an increase in the oxidative capacity of the tissue. That the changes seen in the muscle mitochondria are truly the result of an adaptive change related to cold-acclimation is indicated by experiments in which oxytetracycline was administered to rats exposed to cold. As mentioned previously, oxytetracycline is an inhibitor of mitochondrial protein synthesis and can be used to prevent the development of the enhanced calorigenic response to noradrenaline (see Part A, Section 5) and the accompanying changes in the brown adipose tissue. Figure 10 (Himms-Hagen et al., 1975) shows that the increase in number of muscle mitochondria does not take place in rats treated for two weeks with oxytetracycline. Furthermore, if treatment is commenced on rats already cold-exposed for two weeks and therefore possessing the increased number of mitochondria, the number of mitochondria will decline back to the levels seen in the warm-acclimated rat.

These experiments indicate a role for mitochondrial protein synthesis in cold-acclimation. Further support for this comes from experiments of

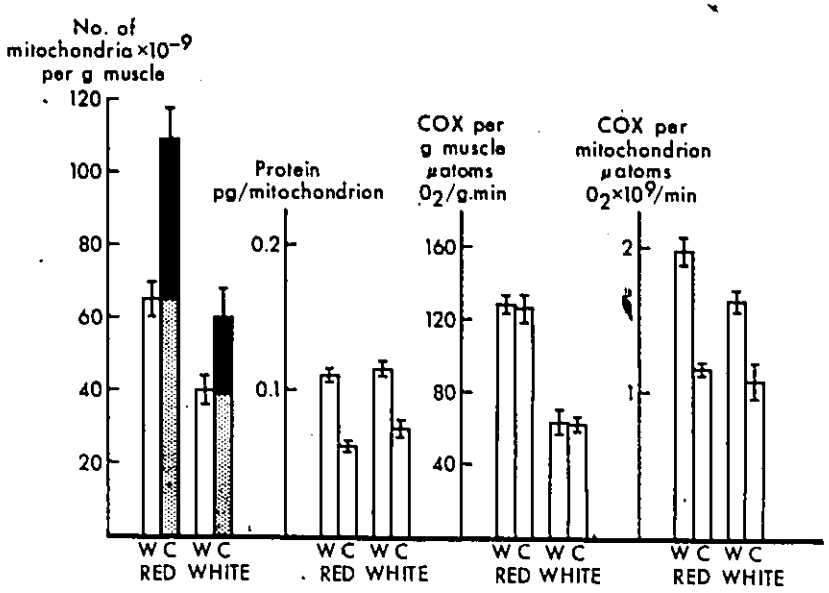

Figure 9. Number of Muscle Mitochondria, Protein and Cytochrome Oxidase (COX) Content of Muscle Mitochondria and Cytochrome Oxidase Content of Muscle in Warm-Acclimated (W) and Cold-Acclimated (C) Rats

Values are shown for red and white portions of the semitendinosus muscle of warm-acclimated and cold-acclimated rats. Rats had lived at their temperature of acclimation for four months and weighed 560± 4.9g (warm-acclimated) and 409.9± 5.6g (cold-acclimated). Each bar represents the mean of 4 animals. Note the increase in the number of mitochondria per gram of red and white muscle and the decrease in the protein and cytochrome oxidase content of a single mitochondrion in both red and white muscle of cold-acclimated rats. Cytochrome oxidase was assayed at 37° C.

(From Himms-Hagen et al., 1975)

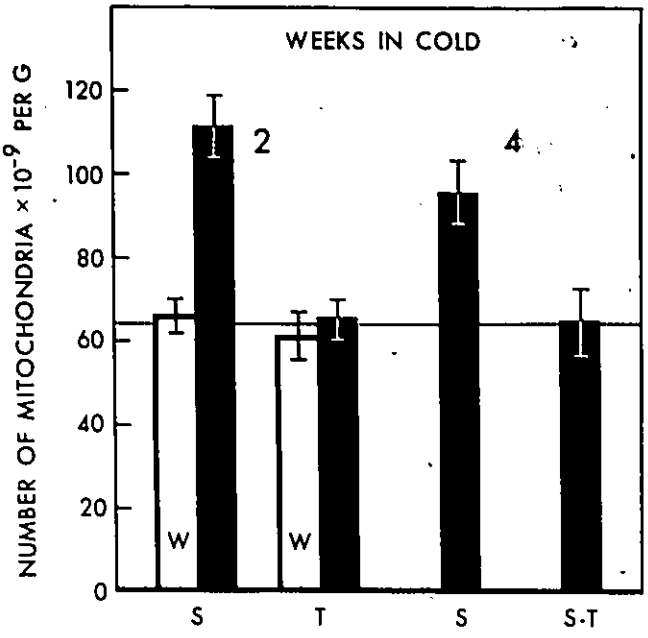

Figure 10. Effect of Treatment with Oxytetracycline on the Cold-Induced Increase in Number of Mitochondria in Skeletal Muscle

Rats living in the cold (black bars) were injected with saline (S) or oxytetracycline (T) as described in the legend to Figure 7. After 2 weeks some of the saline-treated rats received injections of oxytetracycline for a further 2 weeks (group S-T), the remainder continuing to receive injections of saline (group S). Control warm-acclimated rats living at room temperature (W) were similarly injected with either saline or oxytetracycline for 2 weeks. Values are shown for cold-acclimated and warm-acclimated rats at 2 weeks (2) and for cold-acclimated rats only at 4 weeks (4). Note that the increase in number of mitochondria in the muscle of the cold-acclimated rat after two weeks in the cold is prevented by the treatment with oxytetracycline and that the increase disappears when the treatment with oxytetracycline is started only when the rat has already lived in the cold for two weeks. Bars represent mean values for 5 rats (at 2 weeks) or for 3 rats (at 4 weeks). (From Himms-Hagen et al., 1975)

Bukowiecki and Himms-Hagen (1971) in which the half-lives of different groups of mitochondrial proteins were measured (Fig. 11). A greater turnover of certain groups of mitochondrial proteins was observed in mitochondria from brown adipose tissue and muscle, known sites of NST, but not in liver and kidney where little if any NST takes place. Further work is now being carried out in an attempt to separate and identify the individual proteins in these groups that change in the cold-acclimated rat (Himms-Hagen, unpublished).

The discovery of morphological alterations and differences in protein metabolism in association with cold-acclimation has led to the search for other biochemical differences in these mitochondria that might provide a clue to the mechanism of nonshivering thermogenesis. Isolated muscle mitochondria from cold-acclimated rats have normal respiratory control ratios and ADP/O ratios (Fig. 12, Himms-Hagen et al., 1975; Hbous, 1976). The only difference seen is an increased rate of state 3 (ADP-stimulated) respiration with various substrates and smaller increases in state 4 rates. This means that muscle mitochondria have higher respiratory capacities in the cold-acclimated rat but control of respiration rate appears to be unaltered. Muscle mitochondria thus appear not to be uncoupled or loosely coupled as are brown adipose tissue mitochondria, although it is possible, of course, that factors responsible for loose coupling in vivo are not present under in vitro incubation conditions. Furthermore, measurements in Fig. 12 were made in the presence of albumin which could possibly mask differences in the state of coupling. No comparison of the sensitivity of muscle mitochondria from warm- and cold-acclimated rats to fatty acids has yet been made. The results argue against, but do not completely rule out, a mode of heat pro-

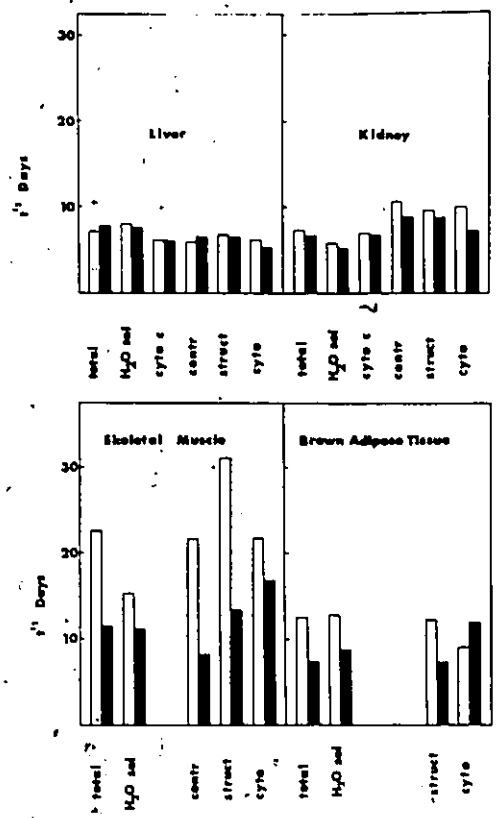

Figure 11. Half-Lives of Different Groups of Mitochondrial Proteins of Liver, Kidney, Interscapular Brown Adipose Tissue, and Skeletal Muscle of Warm-Acclimated and Cold-Acclimated Rats

The half-life (in days) is given for total mitochondrial proteins and for five different groups of mitochondrial proteins (water-soluble, cytochrome c, contractile, structural, other cytochromes) in tissues of warm-acclimated (open bars) and cold-acclimated (black bars) rats. Note that the names used for the different mitochondrial fractions do not imply their function; the fractions are isolated on the basis of solubility properties and each one is comprised of a variety of different proteins.

(From Bukowiecki & Himms-Hagen, 1971)

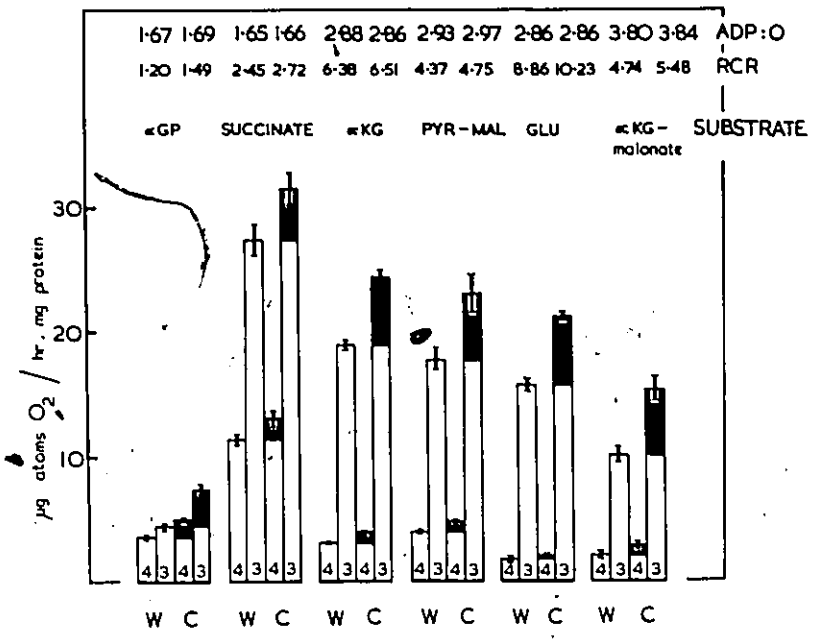

Figure 12. Respiration of Mitochondria Isolated from Mixed Muscle of Warm-Acclimated (W) and Cold-Acclimated (C) Rats

Mitochondria were isolated after proteolytic digestion of mixed leg muscle. They were finally suspended in a medium containing 0.21 M mannitol, 0.07 M KCl, 0.007 M EDTA, 0.45 M sucrose, 0.005 M $MgCl_2$, and 0.5 mg/ml mitochondrial protein. Respiration was measured in the presence of one of the following substrates: 5 mM α-glycerophosphate, 5 mM succinate, 10 mM α-ketoglutarate, 10 mM pyruvate + malate, 10 mM glutamate, 10 mM α-ketoglutarate + 10 mM malonate. State 3 respiration was measured in the presence of 0.2 mM ADP (α-glycerophosphate or succinate) or 0.4 mM ADP (all other substrates); state 4 respiration is the rate after stimulation by ADP has ceased. ADP/O ratios and respiratory control ratios (RCR) are shown at the top of the diagram. Each bar is the mean of values from 9 rats (for α-glycerophosphate and succinate) or 4 rats (all other substrates). The shaded portion of the bars represents the increase in respiration rates seen in the mitochondria from cold-acclimated rats (C) compared to warm-acclimated controls (W). Rates were measured at 37° C.

(From Himms-Hagen et al., 1975)

duction in muscle involving loosely coupled mitochondria.

The levels of various mitochondrial and cytoplasmic enzymes have also been compared in cold- and warm-acclimated rats (Hbous, 1976). The specific activity of mitochondrial α-glycerophosphate dehydrogenase is increased by 60%, while that of malate dehydrogenase is decreased by 24%. The activities of a variety of other mitochondrial and cytoplasmic enzymes are unchanged. One interesting enzyme change that was found was a 35% increase in the adenine nucleotide translocase in the cold-acclimated rat (Hbous, 1976). This system is responsible for transferring ADP into the mitochondrion to the coupling sites for oxidative phosphorylation and carrying the resulting ATP back into the cytosol. Such an increase might be significant because it would provide a means of increasing the rate of ADP supply to the coupling sites and would therefore lead to an increased respiration rate in the cold-acclimated rat, assuming the mitochondria are fully coupled and that substrate and oxygen supply are not rate-limiting. The increased activity of the adenine nucleotide translocase is a likely explanation for the elevated state 3 respiration rates in cold-acclimated rats discussed previously.

A role for the Na^+K^+ATPase in nonshivering thermogenesis in the muscle of cold-acclimated animals has also been suggested. Stevens & Kido (1974) showed that muscle slices from cold-adapted mice have an elevated oxygen consumption which is largely ouabain-sensitive. Increased ouabain-sensitive respiration rates have been noted in diaphragm muscle from cold-exposed and cold-acclimated hamsters (Horwitz, 1975) and rats (Mokhova & Zorov, 1973). Whether these effects are actually related to cold-acclimation as it is commonly understood is questionable. First, it is difficult to understand why a tissue from a cold-acclimated animal should show an increased respira-

tion rate in the absence of noradrenaline since the basal metabolic rates of these animals are not elevated. Secondly, the effect is seen in animals subjected to both short-term and chronic cold-exposure. This is understandable in hamsters, which are hibernators and always possess the adaptation for NST whether cold-exposed or not (Vybiral & Jansky, 1974), but in rats and mice NST takes several weeks to develop. One would not expect to see increased ouabain-sensitive respiration rates in the short-term cold-exposed rat or mouse. A role, then, for the Na^+K^+-ATPase in nonshivering thermogenesis in muscle based on these observations is still largely tenuous.

Preliminary experiments have been done on the activity of the Na^+K^+-ATPase in isolated sarcolemma from warm- and cold-acclimated rats (Cerf & Himms-Hagen, unpublished). The activities of both the Na^+K^+-ATPase and the Mg^{2+}-ATPase increase approximately 30% in the cold-acclimated rat. Work is currently underway to test the sensitivity of the Na^+K^+-ATPase to noradrenaline and other agents.

Research so far has shown that the adaptive changes that take place during cold-acclimation lead to alterations in the muscle mitochondria. This is reflected in an increased turnover of certain mitochondrial proteins and results in a repackaging of the mitochondria so that they become more numerous and smaller in size. This alteration in mitochondrial form is presumed to lead to alterations in mitochondrial function associated with noradrenaline-induced thermogenesis. Even though some biochemical differences have now been found, the mechanism of NST in muscle is still uncertain.

PART C: MITOCHONDRIAL CALCIUM TRANSPORT AND ITS POSSIBLE ROLE IN NONSHIVERING THERMOGENESIS

The previous two parts of the literature review have outlined the

characteristics of nonshivering thermogenesis and cold-acclimation as well as current concepts of the biochemical mechanisms of NST. The purpose of this part is to introduce the concept of mitochondrial calcium transport as a contributing factor in the control of respiration in mitochondria and to propose a role for this process in nonshivering thermogenesis. First, however, it is necessary to describe the characteristics of mitochondrial calcium transport and to review its physiological importance.

1. Mitochondrial Calcium Transport

(a) General Characteristics

Early studies on the effects of calcium on mitochondrial respiration indicated that it acted as an uncoupling agent (Lehninger, 1949) or that it caused deleterious effects by binding in large amounts to mitochondria during isolation (Slater & Cleland, 1953). Siekevitz and Potter (1953) showed that the addition of calcium to respiring mitochondria would stimulate the rate of respiration. Using lower calcium concentrations than previous workers, Chance (1956) found that calcium-stimulated respiration showed state 4 to state 3 transitions very similar to those induced by ADP. He concluded that calcium did not act merely as an uncoupling agent as Lehninger had earlier suggested, but that its reaction with mitochondria was linked stoichiometrically with the respiratory chain. This work was verified and extended by research carried out in the 1960's, principally by Lehninger and coworkers.

The uptake of calcium by mitochondria is an energy-linked process requiring the presence of either respiratory substrate or ATP (see review by Lehninger et al., 1967). Substantial amounts of calcium (greater than about 100 ng-atoms calcium/mg mitochondrial protein) may be taken up only if a

permeant anion such as phosphate is present. This process is referred to as matrix loading because the accumulated calcium appears in the mitochondrial matrix as insoluble amorphous calcium phosphate granules (Lehninger, 1972, 1974a). A special case of matrix loading, called massive loading, can take place if respiratory substrates, ATP or ADP, Mg^{2+} and phosphate are present. Under these conditions over 3000 ng-atoms Ca^{2+}/mg mitochondrial protein may be taken up (Vasington & Murphy, 1962). Such massive loading, however, causes irreversible damage to oxidative phosphorylation and respiratory control mechanisms and gives rise to morphological changes in the mitochondrial structure. It is therefore more useful to study calcium transport processes in mitochondria by using much lower calcium concentrations in the incubation medium.

Mitochondria can still bind calcium in the absence of a permeant anion although the capacity is limited to about 100 ng-atoms Ca^{2+}/mg mitochondrial protein. This type of energy-dependent binding has been called membrane loading (Lehninger, 1972, 1974a) because calcium becomes bound to sites on the mitochondrial inner membrane rather than being deposited into the matrix.

Calcium uptake shows certain stoichiometric relationships with the electron transport chain. For each energy-conserving site, two calcium ions are accumulated (Ca^{2+}:site = 2). This means that the extra oxygen consumption induced by the uptake of two calcium ions is equivalent to that induced by one molecule of ADP (Rossi & Lehninger, 1964; Chance, 1965). It follows that Ca^{2+}:ADP = 2 and Ca^{2+}:O = 2, 4 or 6 depending on the respiratory substrate. If phosphate is present, it is also taken up, with the ratio Ca^{2+}:Pi varying from 1.7-2.0 depending on incubation conditions (Lehninger et al., 1967). Calcium accumulation is also accompanied by proton ejection,

with $H^+:Ca^{2+}$ depending upon incubation conditions (Lehninger et al., 1967); the most recent measurement of this ratio under controlled conditions has shown that $H^+:Ca^{2+} = 2$ (Brand, Chen & Lehninger, 1976). The membrane loading of calcium consists of two phases: a very rapid, early "superstoichiometric" phase in which Ca^{2+}:site ratios and $H^+:Ca^{2+}$ ratios are much higher than normal, and a slower phase showing the usual stoichiometric relationships (Reynafarje & Lehninger, 1974; Lehninger, 1974a).

Another important characteristic of mitochondrial calcium uptake is that it usually takes precedence over ADP phosphorylation. If calcium and ADP are added simultaneously to respiring liver mitochondria, the calcium will be accumulated before ADP phosphorylation begins (Rossi & Lehninger, 1964). In heart mitochondria, however, calcium uptake does not take precedence over ADP phosphorylation, the two processes occurring with equal affinities (Jacobus et al., 1975).

(b) Mechanism of Calcium Uptake

Although mechanisms of calcium uptake involving a direct chemical link to the electron transport chain have been suggested in the past (eg. Chance, 1965), present evidence favors a carrier-mediated system separate from the respiratory chain. A model suggested by Lehninger (1972, 1974a) consists of a passive electrogenic uniport, which means that calcium is free to cross the membrane without the opposite movement of a counter-ion in response to a concentration gradient. Energy supplied by electron transport, however, is necessary to drive the uphill transport of calcium into the mitochondrion under normal conditions, since the calcium concentration of the matrix is higher than that of the cytosol.

It is generally agreed that electron transport generates a negative-inside electrochemical proton gradient across the mitochondrial inner membrane, although the mechanism of this process is uncertain. It could result from

the vectorial arrangement of H^+-yielding and H^+-extracting reactions of electron transport (the chemiosmotic hypothesis), or from vectorial hydrolysis of a high-energy chemical intermediate produced by electron transport (the chemical coupling hypothesis). In any event, this proton gradient is considered to be the ultimate driving force for energy-linked calcium uptake.

Lehninger (1974b) has shown that the only anions capable of supporting matrix loading of calcium are those which enter the mitochondrion as protonated species and which are capable of yielding a proton to the excess hydroxyl ions generated by electron transport that are present inside the inner membrane. These include anions such as phosphate, acetate, bicarbonate, arsenate and butyrate. On the other hand, anions such as nitrate, thiocyanate, and chloride, which cannot donate protons, will not support matrix loading. Lehninger postulates that proton-donating anions enter the matrix first in response to the internal alkalinity produced by electron transport. A negative-inside transmembrane potential due to the transported anions builds up and this is the immediate pulling force for bringing the calcium across the inner membrane. The accumulated calcium may then enter the matrix as a salt of the permeant anion.

Mitochondria may also take up calcium by a different mechanism in the absence of permeant anions by respiration-dependent membrane loading. The capacity for this process is limited to less than 100 ng-atoms Ca^{2+}/mg protein - about the same as passive, low affinity binding of calcium that occurs in the absence of respiration. What distinguishes membrane loading from passive binding is its great rapidity, high affinity and unusual stoichiometry. Respiration is thought to cause release of protons from certain membrane binding sites, resulting in a conformational change that increases greatly the affinity of the binding sites for calcium (Lehninger, 1972; Reynafarje & Lehninger, 1974). The binding sites for membrane loading are probably not

the same as the calcium carrier that is responsible for matrix loading. The early, rapid superstoichiometric phase of membrane loading has recently been shown to be supported by endogenous mitochondrial ATP, the supply of which is dependent upon state 4 respiration (Brand & Lehninger, 1975). The second, slower phase of membrane loading takes place after the exhaustion of the ATP supply, is stoichiometric with electron transport, and stimulates respiration.

It is important, then, to distinguish between the two types of energy-linked calcium binding seen in mitochondria. The first type, membrane loading, has a high affinity but limited capacity, occurs very rapidly, is freely reversible, and shows superstoichiometry. The second type, matrix loading, requires a permeant anion, has a high affinity and very great capacity, is less freely reversible, and shows normal stoichiometry with electron transport.

(c) Evidence for a Calcium Carrier

The existence of a specific calcium carrier molecule or system in the mitochondrial inner membrane is supported by several lines of evidence that will be reviewed briefly here.

The energy-linked cation uptake process in mitochondria is highly specific for calcium (see Lehninger et al., 1967). Strontium and manganese ions are also taken up but to a much smaller extent. Magnesium is not transported at all by liver mitochondria, but is accumulated by heart mitochondria. Quite high concentrations of magnesium are required in the incubation medium, however, and the uptake process differs in several respects from calcium transport (Crompton, Capano & Carafoli, 1976). Energy-linked potassium uptake also occurs in mitochondria, but is extremely slow unless ionophores such as valinomycin are present (Lehninger et al., 1967).

The affinity of mitochondria for calcium uptake is very high, with measured Km's (the concentration of calcium at which uptake rate is half-maximal) ranging from less than 1 μM to about 5 μM (Reynafarje & Lehninger, 1969; Carafoli & Azzi, 1972; Reed & Bygrave, 1975a; Spencer & Bygrave, 1973). However, Scarpa and Graziotti (1973) observed a somewhat higher Km of 92 μM for rat heart mitochondria.

Reynafarje and Lehninger (1969) observed that in respiration-inhibited liver mitochondria two classes of binding sites are present, one class having a high affinity for calcium and the other a much lower affinity. The high affinity sites (Km, the concentration of calcium required to half-saturate the sites, = 0.025 μM) are present in low concentrations in the mitochondrial inner membrane (about 1 ng-atom Ca^{2+} bound/mg protein) and are thought to represent the calcium carrier. The low affinity sites (Km≈100 μM) are more numerous (40 ng-atoms/mg) and most likely represent the passive binding of calcium to membrane phospholipids. The high affinity sites are present in mitochondria from a variety of vertebrate tissues and species, but significantly are absent from yeast and blowfly flight muscle mitochondria (Carafoli & Lehninger, 1971), and most higher plant mitochondria (Chen & Lehninger, 1973). Because the mitochondria from those species that lack the high affinity sites show very little energy-linked calcium-accumulating ability, it is possible that the high affinity sites represent a genetically determined calcium carrier molecule or component of the calcium transport system. However, blowfly flight muscle mitochondria have recently been found to have a normal calcium transport system (Bygrave, Daday & Doy, 1975). Also, several groups of workers have questioned the existence of high-affinity binding sites and have criticised the identification of these sites with

the calcium carrier (Mela & Chance, 1969; Akerman, Saris & Jarvisalo, 1974; Southard & Green, 1974; Reed & Bygrave, 1974a). They argue that high-affinity binding is merely the result of residual energy-linked uptake due to incomplete inhibition of respiration. The status of the high-affinity calcium binding sites is thus uncertain at the present time.

Another characteristic of a carrier-mediated transport process is saturation kinetics. This has been observed for mitochondrial calcium uptake by many workers. Interestingly, graphs of calcium uptake rate versus calcium concentration are sigmoidal rather than hyperbolic, indicating cooperativity (Spencer & Bygrave, 1973; Scarpa & Graziotti, 1973; Reed & Bygrave, 1975a). Possibly the binding of one calcium ion to the carrier enhances its affinity for binding a second calcium ion.

Many carrier-mediated processes are blocked by low concentrations of specific inhibitors. Mitochondrial calcium uptake is inhibited by lanthanides (Mela, 1969) and by the histochemical stain ruthenium red (Moore, 1971) in micromolar concentrations. The two substances differ in their mode of inhibition, with La^{3+} inhibiting competitively and ruthenium red non-competitively (Reed & Bygrave, 1974b). Binding studies of these specific calcium uptake inhibitors have shown that the number of inhibitor binding-sites in the mitochondrial membrane is extremely small. This provides convincing evidence that mitochondrial calcium transport is mediated by a small number of specific calcium carrier molecules in the inner membrane.

The ultimate goal of biochemists studying a membrane transport process is the isolation and characterization of its components and reconstitution of these components into a functioning transport system. Although this goal has not been achieved for mitochondrial calcium transport, significant

advances have recently been made. Proteins having a high affinity for calcium have been isolated from mitochondria (eg. Lehninger, 1971; Sottocasa et al., 1972; Gomez-Puyou et al., 1972), the best characterized being that of Carafoli's group (Sottocasa et al., 1972; Carafoli et al., 1973; Prestipino et al., 1974; Carafoli, 1975). This calcium binding factor is easily prepared from rat liver mitochondria by osmotic shock, indicating that it might be a loosely-bound extrinsic membrane protein. It is a highly acidic, water soluble glycoprotein having a molecular weight of 33,000 daltons. It binds 2-3 calcium ions per mole with high affinity in a reaction that is inhibited by La^{3+} and ruthenium red. Recently Carafoli's group has attempted to reconstitute a calcium transport system using the glycoprotein and artificial lipid bilayers (Prestipino et al., 1974). Although the electrical conductance of the bilayer increased after addition of the glycoprotein to the bilayer in a calcium-containing medium, this was not due to the transport of calcium across the bilayer. Carafoli suggested that calcium was required for the incorporation of the glycoprotein into the bilayer, perhaps by neutralizing surface negative charges, and that the increased conductance resulted from enhanced translocation of other ions, possibly protons. It is likely that other components of the transport system exist and that these will have to be found before a functional calcium transport system can be reconstituted.

In addition to the calcium binding proteins that have been isolated, a divalent cation ionophore has been prepared from beef heart mitochondria (Blondin, 1974). This lipophilic substance can induce influx and efflux of calcium and magnesium from mitochondria in much the same manner as the well-known bacterial divalent cation ionophore A23187 (Reed & Lardy, 1972). The

relationship, if any, of Blondin's ionophore to the calcium carrier systems studied by others is not known.

(d) The Release of Calcium from Mitochondria

If calcium is accumulated by mitochondria, it must also be released. The release process, however, has not been as well characterized as uptake. Drahota et al. (1965) showed that mitochondrial calcium is not irreversibly sequestered, but exists in a dynamic steady-state in which efflux of calcium from the mitochondria is counter-balanced by energy-linked accumulation. State 4 respiration is required to maintain this steady-state. Stucki and Ineichen (1974) demonstrated that calcium recycling is responsible for approximately 20% of the energy dissipation during state 4 respiration.

A number of agents have been shown to induce a rapid release of mitochondrial calcium. These include uncouplers and respiratory inhibitors, which interfere with the energy supply necessary to maintain calcium levels within the mitochondrion. The ionophore A23187, a carboxylic antibiotic prepared from Streptomyces chartreusensis, induces release of both calcium and magnesium from mitochondria. Respiration is stimulated because the released calcium is taken up again by the mitochondrion, the net effect being an enhancement of the energy-dissipating calcium flux that takes place naturally under state 4 conditions (Reed & Lardy, 1972).

Calcium chelating agents such as EDTA and EGTA cause net calcium release by a different mechanism, trapping calcium as it is released during recycling and preventing its reuptake (Reed & Bygrave, 1974b). Ruthenium red has been used in the same manner, but in this case the uptake process is blocked and calcium leaks spontaneously out of the mitochondrion (Stucki & Ineichen, 1974; Sordahl, 1975). However, ruthenium red has been shown to have other

effects as well. Rossi, Vasington & Carafoli (1973) observed that ruthenium red induces a rapid and complete release of calcium if it is added to the medium before calcium uptake is complete. Release did not take place if uptake was allowed to go to completion first. Reed & Bygrave (1974b) found that ruthenium red caused no release and in fact it blocked the EGTA-induced efflux of calcium. They believe that this inhibitor binds to the carrier, preventing its operation in either direction. It is uncertain, then, whether ruthenium red blocks only uptake or both uptake and release. It is quite possible that release may occur by other channels as well as by reversal of the carrier, and these channels may be insensitive to ruthenium red. Since ruthenium red binds non-specifically to mucopolysaccharides, it may have other effects on mitochondrial function as well as on calcium transport. This may explain the contradictory results obtained by various workers.

A number of naturally-occuring compounds have been shown to induce release of mitochondrial calcium. These include cyclic AMP, prostaglandins, sodium ion, phosphoenolpyruvate, and free fatty acids.

Borle (1974) was the first to demonstrate that cyclic AMP in low concentrations (2-4 μM) could induce a rapid and complete release of calcium from previously-loaded liver and kidney mitochondria. A smaller effect was also observed in heart mitochondria. This finding was subsequently verified by Matlib and O'Brien (1974). The physiological implications of this discovery will be discussed in more detail in the next section.

Under certain conditions, prostaglandins in micromolar concentrations can cause liver mitochondria to release calcium (Carafoli & Crovetti, 1973; Baum et al., 1973; Malstrom & Carafoli, 1975). The binding of prostaglandins to the mitochondrial membrane is greatly enhanced by the presence of calcium.

The reaction is very sensitive to pH, with a slightly acidic pH of 6.4 being optimal, and permeant anions for calcium uptake must not be present. The effect of prostaglandin binding is to reduce the ability of mitochondria to retain calcium; the uptake process is not affected. Because the state 4 respiration rate after the calcium-induced respiratory jump is stimulated, Malstrom and Carafoli (1975) postulated that prostaglandins act as calcium ionophores, enhancing the cyclic calcium flux across the mitochondrial membrane in a similar manner to ionophore A23187. Interesting as these observations might be, their in vivo significance is uncertain because in vitro calcium release by prostaglandins requires a low, unphysiological pH and a high concentration of prostaglandins.

Sodium ion was shown by Carafoli et al. (1974) to release calcium from heart mitochondria under certain conditions. These include either the exclusion of energy from the system or the presence of ruthenium red in the medium to prevent reuptake of the released calcium. The phenomenon was surprisingly specific for Na^+; other ions such as K^+ had no effect. Liver mitochondria were not sensitive to the calcium-releasing effects of Na^+. The concentration of Na^+ required to cause calcium release was as low as 2 mM provided ruthenium red was present and the amount of calcium bound to the mitochondrion was small. The mechanism of Na^+-induced calcium release is uncertain, but it most likely involves the displacement of calcium from membrane binding sites rather than by discharging calcium from the matrix. When the mitochondria are allowed to bind larger amounts of calcium the percentage released by Na^+ becomes smaller, indicating that only a small portion of calcium is susceptible to the effects of Na^+, perhaps that fraction bound to specific sites on the inner membrane. Because K^+ does not cross

the mitochondrial membrane as freely as does Na^+, it may not be able to reach the binding sites and therefore cannot induce a similar type of release. It is important to stress that in Carafoli's experiments, Na^+ had little effect on respiring mitochondria in the absence of ruthenium red, making extrapolations to in vivo situations rather difficult.

Chudapongse & Haugaard (1973) discovered that phosphoenolpyruvate (PEP) in concentrations of about 1 mM could induce the efflux of calcium from liver and heart mitochondria. Other glycolytic intermediates were without effect. Atractylate, an inhibitor of the adenine nucleotide translocase, and ATP antagonized the effects of PEP. Because PEP can enter the mitochondrion via the adenine nucleotide translocase in exchange for internal adenine nucleotides, Peng et al. (1974) concluded that PEP induces loss of calcium by depleting the mitochondrial adenine nucleotide pool. Phosphate may also be involved in this phenomenon because inhibitors of mitochondrial phosphate transport were also effective in preventing PEP-induced calcium efflux. In a very recent paper, Sul, Shrago and Shug (1976) proved that the adenine nucleotide translocator was responsible for phosphoenolpyruvate entry into heart mitochondria and that the result was adenine nucleotide and calcium efflux. Interestingly, they also found that palmitoyl-CoA could inhibit the adenine nucleotide translocase, thus providing a potential in vivo mechanism for regulating the effects of PEP on mitochondrial calcium movements. The mechanism of calcium release in this case is unknown, although it likely involves matrix calcium phosphate because both phosphate and ATP (or ADP) are required for the precipitation of calcium phosphate salts in the matrix (see Lehninger, 1970). Possibly, depletion of phosphate and adenine nucleotides by phosphoenolpyruvate mobilizes this calcium pool and makes it

available for movement out of the matrix. Because relatively high concentrations of PEP are required to produce these effects in vitro, the physiological significance of these findings is unclear.

Another naturally-occurring group of compounds that can release mitochondrial calcium are the free fatty acids. This is by virtue of their well-known action as mitochondrial uncoupling agents. For example, brown adipose tissue mitochondria may be reversibly coupled and loosely coupled by fatty acids or their CoA esters (see Part B, page 36). It is completely unknown, however, if fatty acids play a role in regulating calcium ion movements in mitochondria in vivo.

In conclusion, it is important to distinguish between the various types of release described in this section. Release caused by agents such as EGTA and ruthenium red results from the prevention of calcium reuptake during the recycling process, and thus may be described as spontaneous or naturally-occurring release. Sodium ion appears to cause release by displacing membrane-bound calcium. Other agents, including uncouplers (DNP, free fatty acids), ionophores (A23187, prostaglandins), respiratory chain inhibitors, and substances whose mechanism of action is less certain (cyclic AMP, phosphoenolpyruvate) discharge a large portion of the matrix calcium.

2. Physiological Significance of Mitochondrial Calcium Transport

The energy-linked uptake of calcium seen in isolated mitochondria is more than just an in vitro curiosity. Within the last ten years, evidence has been accumulating that mitochondria play an important role in regulating the calcium concentration of the cell cytosol, and as a result, participate in controlling a variety of cell functions. The purpose of this section is to outline this aspect of mitochondrial calcium transport.

(a) Distribution of Calcium in Cells

Calcium is compartmentalized within the cell and mechanisms exist for regulating its distribution (see reviews by Borle, 1973; Rasmussen et al., 1975; Berridge, 1975). The concentration of calcium in the cytosol ranges from about 10^{-5}-10^{-7} M; this contrasts with the much higher level of 10^{-3} M found in the extracellular fluid that bathes the cells. It has been known for a long time that much of the total cell calcium is associated with the mitochondrial fraction (Thiers & Vallee, 1957). If all the mitochondrial calcium were in the ionic form, the matrix calcium concentration would be 1-20 x 10^{-3} M, but since most of the matrix calcium exists as insoluble calcium phosphate, the free calcium concentration of the matrix is more likely to be about 10^{-4} M (Rasmussen et al., 1975). In muscle cells, the sarcoplasmic reticulum represents another important calcium store. Microsomal fractions from non-muscle tissues, however, show little calcium-binding ability.

Intracellular levels of calcium are maintained by energy-driven calcium pumps that keep the calcium concentration of the cytosol at low levels. The mitochondria contain an inwardly-directed calcium pump as described in detail in the preceeding section. The plasma membrane is relatively impermeable to calcium in the resting state, but calcium does tend to leak in from the extracellular fluid due to the large concentration gradient for this ion that exists across the membrane. Thus an outwardly-directed calcium pump is present that is most likely equivalent to the calcium-activated ATPase found in the plasma membrane of many cells. In nerve and heart muscle cells, outward extrusion of calcium is linked to the inward movement of sodium ion, in other words, there is a Na-Ca carrier system driven by the

Na^+ gradient across the sarcolemma (Brinley, 1973; Reuter, 1974). Finally, an inwardly-directed calcium ATPase exists in the sarcoplasmic reticulum of muscle cells.

Borle (1972, 1973), using kinetic analysis of influx and efflux of calcium from cultured kidney cells, has shown that the mitochondria function as the largest single internal reservoir of calcium. He estimated that compared to the plasma membrane the mitochondria contribute 97% to the homeostasis of cytoplasmic calcium levels. In muscle this percentage would be less because of the high affinity of the sarcoplasmic reticulum for calcium binding.

In addition to their more commonly known function of energy production within the cell, mitochondria thus act as an intracellular calcium buffer, keeping the calcium concentration of the cytosol at a low level. The importance of this will be made apparent in subsequent discussions.

(b) Calcium as a Second Messenger in Cell Activation

The second messenger hypothesis, in which cyclic AMP participates in a great variety of cellular activation processes, has been one of the most significant and widely studied concepts of modern biochemistry. Briefly, the hypothesis states that the action of hormones (first messengers) upon their membrane receptor sites causes the stimulation of the membrane-bound enzyme adenylate cyclase, which catalyzes the conversion of ATP to cyclic AMP. This compound, the second messenger, activates a class of enzymes known as protein kinases, which in turn bring about phosphorylation of certain key enzymes or other proteins. This protein phosphorylation results in the ultimate expression of the hormone's physiological effect. A full discussion of this hypothesis and numerous examples may be found in the book

by Robison, Butcher and Sutherland (1971).

Many researchers have tended to overemphasize the importance of cyclic AMP and have overlooked the involvement of calcium in many hormonal responses. Rasmussen (1970) pointed out that the actions of many hormones leading to increases in cyclic AMP levels also require the presence of calcium, and he formulated a theory involving calcium and cyclic AMP as second messengers in cellular activation processes. Rather than describe all of these processes in detail, only a brief outline of Rasmussen's hypothesis will be given, followed by a few examples illustrating the role of mitochondrial calcium transport in cell activation. More information about the discussion that follows may be found in articles by Bygrave (1967), Rasmussen (1970), Rasmussen, Goodman & Tenenhouse (1972), Borle (1973), Lehninger (1970, 1974a), Rasmussen et al. (1975) and Berridge (1975).

Rasmussen's general theory of cell activation may be described as follows. The binding of a hormone to its receptor site on the plasma membrane of a cell has two simultaneous effects: first, adenylate cyclase is activated and the concentration of cyclic AMP in the cytosol is increased; secondly, the permeability of the plasma membrane to calcium is increased and calcium leaks into the cytosol from the extracellular fluid. The increased cytosolic level of cyclic AMP has at least two effects. The first is the activation of one or more protein kinases which catalyze the phosphorylation of certain proteins, leading to the expression of the hormone's effect. This protein phosphorylation leads, for example, to the activation or inhibition of a regulatory enzyme in a metabolic pathway, or the enhancement of protein synthesis at the transcriptional or translational level. The second effect of cyclic AMP is to alter the distribution of calcium levels within the cell. In some cases it causes the net release of mitochondrial

calcium. This effect, plus the increased plasma membrane permeability to calcium caused by the primary stimulus, results in the elevation of cytosolic calcium concentrations. In some systems cyclic AMP itself can also stimulate the influx of extracellular calcium through the plasma membrane. In muscle, cyclic AMP stimulates the uptake of calcium by the sarcoplasmic reticulum. While cyclic AMP has the ability to modulate calcium distribution, calcium can function as a negative feedback inhibitor by decreasing cyclic AMP levels through inhibition of adenylate cyclase and activation of cyclic AMP phosphodiesterase. Thus a complex network of feedback relationships exist between the two second messengers.

How does calcium function as a second messenger? Excitation-contraction coupling in muscle is probably the best known example, with calcium acting as the signal for initiating the contractile process. Calcium also plays a role in stimulus-secretion coupling by enhancing the movement of microfilaments that function in exocytosis in secretory cells. This is thought to occur during insulin release in the endocrine pancreas. The permeability of cell membranes to certain ions is enhanced by calcium; this can be seen in the fly salivary gland where the elevated cytosolic calcium levels induced by serotonin increase the permeability of the membrane to chloride. Finally, the activity of a number of important enzymes is sensitive to calcium. Good examples of these are pyruvate kinase (Bygrave, 1967; Meli & Bygrave, 1972), pyruvate dehydrogenase (Pettit, Roche & Reed, 1972; Severson et al., 1974), α-glycerophosphate dehydrogenase (Carafoli & Sacktor, 1972; Bukowiecki & Lindberg, 1974) and adenine nucleotide translocase (Spencer & Bygrave, 1972). Another enzyme activated by calcium is muscle phosphorylase b kinase, which is involved in the glycogen cycle, catalyzing the formation of glycogen

phosphorylase a. This is interesting because a protein kinase (phosphorylase kinase kinase) converts phosphorylase b kinase from an inactive to an active form, and thus is an example of the close relationship between cyclic AMP and calcium.

Because many cellular functions are sensitive to the concentration of calcium and because the mitochondria represent an important system for regulating calcium distribution within the cell, the physiological significance of mitochondrial calcium transport is readily apparent.

(c). Relationships Between Catecholamines, Cyclic AMP and Calcium in Movements in Muscle Cells

A more detailed discussion of the role of calcium in muscle function is desirable at this point because it illustrates many of the principles just described and because it is relevant to the research reported in this thesis.

Mammalian muscle may be divided into three basic types on the basis of physiological, ultrastructural and biochemical considerations. These are: skeletal or striated muscle, responsible for voluntary body movements; smooth muscle, which makes up the involuntary musculature of blood vessels, the gastrointestinal tract, genito-urinary system and respiratory system; and cardiac muscle, which of course constitutes the heart. Skeletal muscle may be further subdivided into three structurally and biochemically distinct fibre types: red (fast-twitch oxidative glycolytic), white (fast-twitch glycolytic) and intermediate (slow-twitch oxidative) (see Gauthier, 1969; Peter et al., 1972). Often one sees research papers in which only two types of skeletal muscle are mentioned, namely red (slow) and white (fast) muscle.

The general mechanism of muscle contraction has been outlined in recent reviews (Ebashi & Endo, 1968; Bianchi, 1973; Ebashi, 1976), and will only

be briefly outlined here to emphasize the role of calcium in the process. Transmission of the nerve impulse via acetylcholine to the muscle sarcolemma causes depolarization and generation of the action potential. This is transmitted to the T-tubules, invaginations of the surface membrane lying in close proximity to the terminal cisternae of the sarcoplasmic reticulum. The action potential is somehow transferred to the terminal cisternae resulting in the release of calcium into the sarcoplasm. The mechanism of this process is not certain, but it could involve a direct electrical transmission from the T-tubule to the sarcoplasmic reticulum causing release of calcium, or it may involve release of a small amount of calcium from the T-tubular membrane which then triggers the release of larger amounts of calcium from the terminal cisternae. Under resting conditions, the calcium concentration of the sarcoplasm is low (10^{-7} M), and interaction between the actin and myosin filaments is prevented due to the presence of an inhibitory protein complex (troponin plus tropomyosin) bound to actin. Release of calcium from the sarcoplasmic reticulum during excitation elevates the calcium concentration of the sarcoplasm to 10^{-6}-10^{-5} M. Calcium ions bind to troponin, inducing a conformational change in the protein that negates the suppressor effect of the troponin-tropomyosin complex. The actin-myosin interaction can then occur, deriving energy from ATP, and the result is muscle contraction. Relaxation occurs when the excess calcium is actively pumped back into the sarcoplasmic reticulum, permitting the contractile proteins to return to their resting condition.

The molecular mechanism of contraction appears to be basically the same in all types of muscle, but differences exist in the source of calcium required to initiate contraction and the method of sequestering the calcium during relaxation. In addition to the sarcoplasmic reticulum, the sarcolemma

and mitochondria must also be considered as participants in controlling calcium movements during contraction-relaxation. This is especially so in muscles that lack a well-developed sarcoplasmic reticulum, such as red skeletal muscle, smooth muscle, and cardiac muscle (Bianchi, 1973). Other aspects of calcium and muscle function will now be examined, looking at each type of muscle separately.

(i) Skeletal Muscle

Contraction of skeletal muscle involves the entry of a small amount of calcium from the extracellular fluid and the release of much larger amounts of calcium from the sarcoplasmic reticulum as discussed above. Red skeletal muscle, however, differs from white muscle in that more mitochondria are present and the sarcoplasmic reticulum is more sparse. It has been suggested that in red muscle, mitochondria are important in controlling calcium movements during contraction and relaxation (Lehninger, 1970, 1974a). There is some experimental evidence for this. Patriarca and Carafoli (1969) found that after injection of radioactive calcium (^{45}Ca) into rabbits, the mitochondria of red muscles contained a considerably greater percentage of ^{45}Ca than did the sarcoplasmic reticulum. Mitochondria and sarcoplasmic reticulum from white muscle, however, contained about equal levels of ^{45}Ca. This experiment was interpreted as indicating that the mitochondria are more important than the sarcoplasmic reticulum in regulating calcium movements in red muscle, but it could also mean that more calcium was bound to the mitochondria during the isolation procedure. Ruthenium red and respiratory inhibitors block relaxation of skinned red muscle fibres but not white fibres, again suggesting a role for mitochondria in the contraction-relaxation of red muscle (Lehninger, 1974a).

In addition to its role in excitation-contraction coupling, calcium is associated with metabolic events in muscle (see Himms-Hagen, 1972b). The increased cytosolic levels of calcium that occur during contraction can enhance the breakdown of glycogen through activation of phosphorylase b kinase. Thus an energy-requiring process, contraction, is linked to an energy-producing pathway through the common second messenger calcium. The catecholamines can also enhance glycolysis by phosphorylase b kinase activation, but this occurs via the cyclic AMP-stimulated phosphorylase kinase kinase.

Catecholamines also have a rather poorly-understood effect on contractile processes in skeletal muscle (see review by Tomita, 1975). Adrenaline will enhance neuromuscular transmission, possibly through a cyclic AMP-mediated acceleration of acetylcholine release (Goldberg & Singer, 1969). Cyclic AMP could act by increasing the permeability of neurosecretory cells to calcium, which is known to regulate the release of neurotransmittors (Berridge, 1975). Catecholamines also affect the muscle cell more directly by influencing the tension developed during stimulation. In fast-contracting muscles, twitch tension is increased and the rate of relaxation is decreased by catecholamines, while the reverse occurs in slow-contracting muscles (Bowman & Raper, 1967). It is unknown whether these events involve calcium or cyclic AMP.

Changes in membrane potentials have been observed in skeletal muscle treated with catecholamines. Various workers have reported catecholamine-induced sodium efflux and/or hyperpolarization of muscle preparations (Evans & Smith, 1973; Dockry, Kernan & Tangney, 1966; Vyskocil, Moravec & Melichar, 1975). Depolarization of hamster muscle cells after noradrenaline injection into the cells *in vivo* was noted by Teskey, Horwitz and Horowitz (1975).

Bressler, Phillis and Kozachuk (1975) found that noradrenaline hyperpolarizes frog skeletal muscle in a low potassium medium but depolarizes it in a high potassium medium. These conflicting reports are undoubtedly due to differences in expermental conditions and type of muscle fibre used. It is known, for example, that resting membrane potentials differ among the various muscle fibre types (Campion, 1974). The exact mechanism of catecholamine action on ion fluxes is not known, although stimulation of the Na^+K^+-ATPase has been suggested (Bressler et al., 1975; Hays et al., 1974; Evans & Smith, 1973; Dockry et al., 1966).

(ii) Smooth Muscle

Contraction and relaxation of smooth muscle is regulated by calcium levels in the cytosol in a similar manner to skeletal muscle. In general, however, the sarcoplasmic reticulum of smooth muscle is not very well developed and so a greater role for the sarcolemma and mitochondrial calcium pumps in the contraction-relaxation cycle is likely.

Contraction of smooth muscle induced by acetylcholine involves increases in cytosolic calcium due to increased entry of calcium from the extracellular fluid and increased release of calcium from intracellular stores (sarcoplasmic reticulum and possibly mitochondria) (Rasmussen et al., 1972; Berridge, 1975). The relative importance of the calcium source varies with the type of smooth muscle - the lower the amount of sarcoplasmic reticulum, the more important the extracellular source. Mitochondria have been implicated in the contraction-relaxation cycle of the human myometrium (Batra, 1973); these organelles were found to have a greater calcium binding activity than microsomes from this type of smooth muscle.

Catecholamines cause the relaxation of smooth muscle by increasing cyclic

AMP levels in the cell. Cyclic AMP stimulates the uptake of calcium by the plasma membrane and the sarcoplasmic reticulum, thereby lowering the cytosolic calcium concentration and causing relaxation.

(iii) Cardiac Muscle

Heart muscle differs from skeletal muscle in that it has a poorly-developed sarcoplasmic reticulum and requires external calcium for contraction. Calcium enters during the action potential and, in addition to that released from the sarcoplasmic reticulum, catalyzes the contractile process (Reuter, 1974). Relaxation results from removal of calcium by the usual mechanism of calcium uptake by the sarcoplasmic reticulum, and also by extrusion from the cell by a sarcolemmal Na-Ca exchange system (Reuter, 1974). Various workers have argued in favor of the mitochondria participating in the contraction-relaxation cycle of heart (eg. Haugaard et al., 1969; Lehninger, 1970, 1974a; Carafoli & Azzi, 1972; Carafoli et al., 1974; Jacobus et al., 1975). Heart muscle contains a great number of mitochondria (40% of the cytoplasmic volume, Lehninger, 1974a) that are situated in close proximity to the myofibrils. The sarcoplasmic reticulum on the other hand is rather sparse in heart compared to fast skeletal muscle, and in amphibian hearts it seems to be lacking altogether. The mitochondria are found to contain most of the radioactivity if ^{45}Ca is injected into an intact animal (Patriarca & Carafoli, 1968) or perfused into contracting rat hearts (Horn, Fyhn & Haugaard, 1971). The affinity of heart mitochondria for calcium is sufficiently high to remove calcium bound to troponin in vitro (Carafoli & Azzi, 1972; Carafoli et al., 1975). On the other hand, Schaffer, Safer and Williamson (1972) argued against mitochondrial involvement in the cardiac contraction-relaxation cycle because rat hearts, perfused anaerobically with oligomycin,

conditions that prevent both respiration and ATP-supported calcium uptake, are still able to beat normally. Scarpa and Graziotti (1973) found that in their assay system the affinity of heart mitochondria for calcium and the rate of calcium uptake was not sufficient to account for the amount of calcium sequestered and released during each beat. However, neither of these groups have taken into account the very rapid superstoichiometric membrane loading of calcium that is known to take place in heart mitochondria (see Section I, page 51). It has been calculated (Jacobus et al., 1975), that heart mitochondria need only to bind 0.5-0.8 ng-atoms calcium/mg protein in order to account for relaxation. This is clearly within the limits of membrane loading (100 ng-atoms/mg protein).

One question that is always raised with respect to mitochondria and the contraction-relaxation cycle of the heart concerns the mechanism of release of calcium from the mitochondria needed to cause contraction. Possibly, depolarization of the T-system could be transferred to the mitochondria resulting in calcium discharge, but this would require some sort of functional contact between the T-system and the mitochondrial membrane. The recent observation by Carafoli's group (Carafoli et al., 1974) that small amounts of sodium can release mitochondrial calcium suggests a mechanism by which sodium influx during the action potential induces calcium release by the mitochondria. During the recovery phase, when sodium is pumped out of the cell, mitochondria take up the calcium again and relaxation results. This mechanism is still highly speculative however, and the role of mitochondria in the cardiac contraction-relaxation cycle is still not firmly established.

Another interesting aspect of heart function is the mechanism of action of inotropic agents. These are substances that increase the force of contraction of the heart, and examples include catecholamines, glucagon, thyroxine and cardiac glycosides. Many inotropic agents stimulate adenylate

cyclase, and so much research has been done on the relationship between cyclic AMP and myocardial contractility (see review by Entman, 1974). Calcium can be expected to be involved in the mechanism of the inotropic response of the heart because of its role in muscle contraction and interrelationships with cyclic AMP.

One way of increasing the force of contraction would be to increase the amount of calcium released and taken up by the sarcoplasmic reticulum during each cycle. Adrenaline (or dibutyryl cyclic AMP) will enhance the entry of calcium from the extracellular fluid into the heart cytoplasm (Meinertz, Nawarth & Scholz, 1973; Reuter, 1974). This extra calcium could be stored in the sarcoplasmic reticulum, thus increasing the quantity of calcium available for release during the next action potential. Furthermore, cyclic AMP has been shown to stimulate the uptake of calcium by cardiac microsomes (Epstein, Levey & Skelton, 1971; Shinebourne & White, 1970; Kirchberger et al., 1972). Cyclic AMP has two other effects that would result in a shift of calcium to the sarcoplasmic reticulum. First, it inhibits the outwardly-directed calcium pump in the sarcolemma (Dietze & Hepp, 1972) and the Na-K ATPase of the sarcolemma (Limas, Notargiacomo & Cohn, 1973). The latter effect will inhibit calcium efflux because it will reduce the sodium gradient that drives the Na-Ca carrier system. Secondly, cyclic AMP causes the release of calcium from heart mitochondria (Borle, 1974). This scheme provides one explanation of how cyclic AMP could modulate the inotropic action of catecholamines by adjusting the intracellular distribution of calcium.

This part of the literature review has attempted to provide an overview of the complex interrelationships that exist within cells, and has stressed the importance of calcium and cyclic AMP as second messengers in cellular activation. It has also been shown that the ability of mitochondria to take

up and release calcium is intimately involved in regulating the internal ionic environment of the cell.

3. Mitochondrial Calcium Transport: Possible Role in Nonshivering Thermogenesis in Muscle

As discussed in Part B, Section 3(b) of the literature review, the mechanism of nonshivering thermogenesis in muscle is still not understood. In view of the increasing evidence implicating calcium in a wide variety of cellular processes, it would be very interesting to see whether calcium movements in muscle cells play any role in noradrenaline-mediated heat production. The purpose of this section is to present a hypothesis, based on the facts described earlier in the literature review, that might explain nonshivering thermogenesis in muscle.

The basic tenet of the proposed hypothesis is that nonshivering thermogenesis involves a noradrenaline-induced increase in calcium-associated energy-dissipation in skeletal muscle mitochondria. Possible ways that this might occur are outlined in Figure 13. Noradrenaline binding to its receptor on the plasma membrane results in an increased level of a calcium releasing agent (eg. cyclic AMP, Na^+, PGE, PEP, FFA) in the sarcoplasm. The releasing agent then enhances the efflux of calcium out of the mitochondria. This increases the availability of calcium for energy-linked reuptake by the mitochondria, thus stimulating respiration, increasing the oxidation of substrates and producing a greater amount of heat. Other potential mechanisms of increasing the calcium concentration of the sarcoplasm would be increased entry of calcium from the extracellular fluid or increased release of calcium from sarcoplasmic reticulum stores.

A mechanism of NST involving mitochondrial calcium transport must satisfy

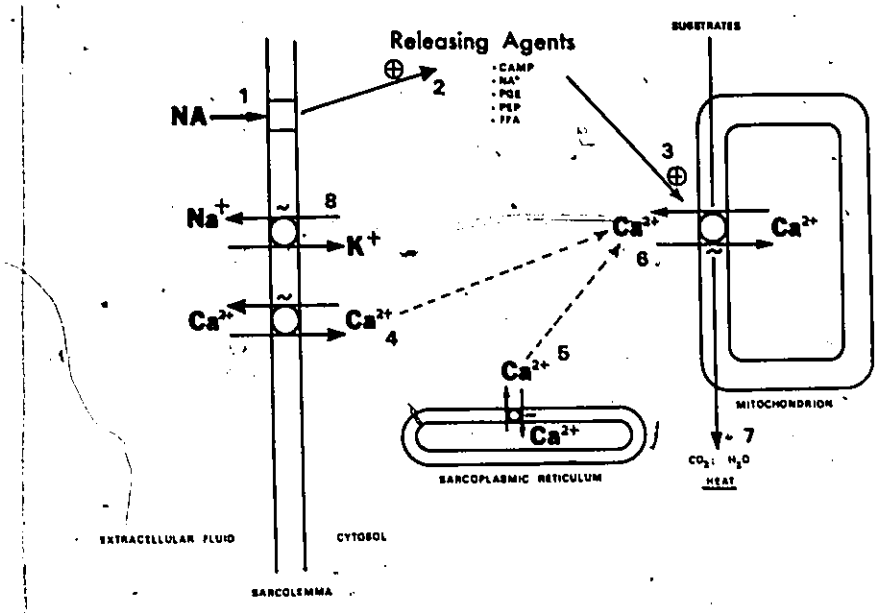

Figure 13. A Hypothetical Mechanism of Nonshivering Thermogenesis in Skeletal Muscle

Noradrenaline acts upon its receptor in the sarcolemma (1), causing an increase in the cytosolic concentration of a releasing agent (2), which in turn enhances calcium efflux from the mitochondrion (3). This makes more calcium available for energy-linked reuptake (6), and respiration is stimulated (7). Other potential sources of calcium are the sarcoplasmic reticulum (5) and the extracellular fluid (4). Also shown is the Na^+K^+ATPase (8), which may be involved indirectly in calcium movements as described in the text.

two basic criteria. First, it must work at a much higher level in the cold-acclimated rat to account for the four-fold increase in metabolic rate seen in cold-acclimated rats infused with noradrenaline. Secondly, there must be a way to turn the mechanism on and off rapidly to account for the facultative characteristic of NST. The first criterion could be satisfied by postulating that in the cold-acclimated rat, more releasing agent is produced by the same amount of noradrenaline. Alternately, the mitochondria could undergo an adaptive change resulting in an increased sensitivity to the releasing agent, that is, in the cold-acclimated rat, the releasing agent induces a greater efflux of calcium from the muscle mitochondria than in the warm-acclimated rat. Another site at which an adaptive change could take place is the uptake portion of the calcium recycling process. By virtue of a greater number or higher affinity of calcium carrier molecules, mitochondria from the cold-acclimated rat could accumulate calcium at a greater rate, thus causing a greater stimulation of oxygen consumption than in the warm-acclimated rat. The second criterion, the rapid switching on and off of NST, could be explained if one assumes that the releasing agent is removed rapidly as soon as the stimulus (noradrenaline) is no longer present.

There are, of course, a number of problems associated with this hypothesis that would have to be solved experimentally before it could be accepted. First of all, the releasing agents mentioned have all been shown to release calcium from isolated mitochondria, but it is uncertain whether they function in the intact animal. The second problem is whether noradrenaline can increase the levels of the various releasing agents within the muscle cell. Cyclic AMP would be an attractive candidate for participating in a noradrenaline-induced enhancement of calcium recycling, because levels

of this compound would be increased by the stimulation of adenylate cyclase by noradrenaline. The concentrations of cyclic AMP required to induce mitochondrial calcium release (Borle, 1974) appear to be within the physiological range. Also, a method for rapid removal of this releasing agent also exists, namely hydrolysis by phosphodiesterase. However, cyclic AMP would be expected to have other actions in muscle as well. In heart, cyclic AMP inhibits the Ca^{2+}-ATPase and $Na^{+}K^{+}$-ATPase of the sarcolemma (Dietze & Hepp, 1972; Limas et al., 1973), but whether this happens in skeletal muscle is not known. Cyclic AMP stimulates the uptake of calcium by sarcoplasmic reticulum in heart (Kirchberger et al., 1972) and in red skeletal muscle but not white muscle (Kirchberger & Tada, 1976). Such an action would tend to make cytosolic calcium unavailable for uptake by the mitochondria and so would be difficult to reconcile with the proposed hypothesis.

Finally, no changes have been observed in the muscle adenylate cyclase system of rats fully acclimated to cold (Himms-Hagen et al., 1975). Noradrenaline would thus not be expected to increase cyclic AMP concentrations to a higher level in the cold-acclimated rat compared to a warm-acclimated rat. One would have to postulate that muscle mitochondria from the cold-acclimated rat are more sensitive to the calcium releasing effects of cyclic AMP.

Of the other known releasing agents, free fatty acid levels would be expected to increase as a result of catecholamine-induced lipolysis (see Garland & Randle, 1963). Little is known, however, about the effects of fatty acids on muscle mitochondrial function in vivo. Muscle mitochondria isolated from cold-acclimated rats, for example, do not show the fatty acid-induced uncoupling seen in brown adipose tissue mitochondria. Phosphoenol-

pyruvate levels possibly could be elevated as a result of enhanced glycolysis caused by noradrenaline, but it seems likely that this compound would be very rapidly metabolized to pyruvate before sufficiently high levels could accumulate to cause mitochondrial calcium release. Prostaglandins, likewise, must be present in high quantities to induce release in in vitro experiments. Interestingly enough, though, there is some evidence that prostaglandins might be involved in cold-acclimation. Cold-stressed rats excrete higher levels of prostaglandin metabolites than controls (Green & Samuelsson, 1971). Rats treated with indomethacin, an inhibitor of prostaglandin synthesis, survive poorly in the cold and show increased excretion rates of noradrenaline (Stjarne, 1972). There appear to be feedback relationships between prostaglandins and catecholamines, that is, sympathetic stimulation causes prostaglandin release, which in turn inhibits release of noradrenaline (see Weeks, 1972). Whether prostaglandins play a direct role in thermogenic mechanisms, however, is not known.

Sodium ion is another calcium releasing agent whose concentration might be regulated by noradrenaline. Two ways in which noradrenaline could increase sodium levels in the cytosol are by depolarizing the sarcolemma (Teskey et al., 1975) or by inhibiting the Na^+K^+-ATPase. The effects of catecholamines on the Na^+K^+-ATPase vary from tissue to tissue, with noradrenaline inhibiting it in liver (Barnabei et al., 1973) and heart (Limas et al., 1973), but stimulating it in brown adipose tissue (Herd et al., 1970) and brain (Godfraind, Koch & Verbeke, 1974). Although Teskey et al. (1975) observed depolarization of skeletal muscle cells by noradrenaline, various other workers have noted sodium efflux and have suggested that noradrenaline or adrenaline stimulates the sodium pump (Dockry et al., 1966; Evans & Smith, 1973; Hays et al., 1974; Bressler et al., 1975). Establishment of a

role for sodium as a calcium releasing agent in the proposed hypothesis will have to await clarification of the effects of noradrenaline on sodium fluxes in skeletal muscle.

Another complication that arises with respect to this hypothesis is the active calcium accumulating ability of the sarcoplasmic reticulum. Any calcium released by the mitochondria could be taken up by the sarcoplasmic reticulum and made unavailable for mitochondrial uptake. The calcium binding constant of isolated sarcoplasmic reticulum is about 1 μM (Meissner, Conner & Fleischer, 1973) which is similar to the affinity of mitochondria for calcium. A second problem is that the amount of calcium released by the mitochondria must not be sufficient to initiate muscle contraction, since nonshivering thermogenesis does not involve muscle movements. One would have to postulate that the noradrenaline-induced increase in calcium recycling is a closely controlled local event occurring at the mitochondrial membrane, and that the calcium that leaks out is reaccumulated rapidly by the mitochondrion and is therefore not made available either for uptake by the sarcoplasmic reticulum or for participation in the contractile process. This type of release would thus be basically different from that occurring as a result of nervous stimulation, in which the action potential causes a rapid and large amount of calcium release from the sarcoplasmic reticulum.

The purpose of the research reported in this thesis is to examine one particular aspect of the proposed hypothesis, namely the calcium transporting properties of muscle mitochondria isolated from cold- and warm-acclimated rats. This is a logical starting point for testing the hypothesis because it is already known that muscle mitochondria undergo morphological and biochemical changes associated with cold-acclimation, changes which have not so

far been associated with any marked change in mitochondrial function. Also, an appreciable amount of research has already been done on the various aspects of mitochondrial calcium transport, and so parameters exist to which muscle mitochondria from cold-acclimated rats can be compared.

The following aspects of mitochondrial calcium transport will be compared in skeletal muscle mitochondria isolated from warm- and cold-acclimated rats:

(i) calcium contents of the mitochondria

(ii) effects of calcium on mitochondrial respiration

(iii) rate of calcium uptake by mitochondria

(iv) effects of various compounds on the release of calcium from mitochondria

(v) measurement of respiration-inhibited high affinity and low affinity calcium binding sites in mitochondria.

An attempt will be made during the discussion of the results of these experiments to correlate the findings with the hypothetical model of non-shivering themogenesis presented in this section.

The ultimate goal of the experiments reported here and others in this laboratory is to explain in biochemical terms the mechanism of nonshivering thermogenesis in cold-acclimated rats. Such an achievement would contribute significantly to our understanding of adaptive processes and hormonal regulatory mechanisms in endotherms.

CHAPTER III: MATERIALS AND METHODS

PART A: MATERIALS

1. Rats

Male white rats of the Holtzmann strain weighing 150-200 g were purchased either from the Holtzmann Company (Madison, Wisconsin), or from Canadian Breeding Laboratories (LaSalle, Quebec). Upon arrival they were kept at room temperature ($27^{\circ} \pm 1^{\circ}C$) for one week in large wire cages containing about 12 rats per cage. Rats were then placed in individual wire cages and divided into two groups: warm-acclimated controls, left at room temperature, and cold-acclimated, placed in a cold room having a temperature of $4^{\circ} \pm 1^{\circ}C$. Rats had free access to food and water, and artificial lighting was maintained 12 hours a day (0600-1800 hr). Rats were weighed weekly and just before each experiment; only rats showing normal growth were used in experiments. Rats were left in the cold room for at least five weeks before use to ensure that full acclimation to cold had been achieved.

2. Chemicals

The following chemicals were obtained from the Sigma Chemical Co.: ATP (adenosine 5'-triphosphate, disodium salt), ADP (adenosine 5'-diphosphate, disodium salt), EDTA (ethylenediaminetetraacetic acid, disodium salt), EGTA (ethyleneglycol-bis-(aminoethylether) N,N'-tetraacetic acid), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), heparin (158 units/mg, sodium salt), mannitol, sucrose, bovine serum albumin (fatty acid free), protease (subtilisin BPN'), imidazole, ruthenium red, PPO (2,5-diphenyloxazole), rotenone, antimycin A, pyruvic acid (free acid and sodium salt), malic acid (free acid and sodium salt), succinic acid, glutamic acid, DL-α-glycero-

phosphoric acid (sodium salt), and Tris (trishydroxymethylaminomethane, acid and base).

Radioactive calcium ($^{45}CaCl_2$, 10-20 mCi/mg) was obtained from New England Nuclear. NCS tissue solubilizer was purchased from Amersham-Searle. Filter-Solv cellulose acetate filter dissolution agent was a product of Beckman Instruments, Inc. Lanthanum chloride ($LaCl_3 \cdot 6H_2O$) and calcium reference solution (1000 ppm, for atomic absorption spectrophotometry) were obtained from Fisher Scientific Co.

Divalent cation ionophore A23187 and prostaglandin E_1 were gifts of Dr. R. Hamill of Eli Lilly and Co. and Dr. J. Pike of the Upjohn Co. respectively.

PART B: METHODS

1. Isolation of Muscle Mitochondria

For the majority of the studies reported in this thesis, muscle mitochondria were isolated by a slight modification of the method reported by Behrens and Himms-Hagen (1976).

Shortly before the experiment, rats were taken to the laboratory from their respective environmentally-controlled rooms, weighed, and killed by decapitation. Hind leg muscles were quickly excised and immersed in cold 0.15 M KCl. Subsequent steps were all performed at 0-4°C. After removal of fat and connective tissue, the muscles were minced with a razor blade into small cubes, and 10 g of the muscle mince was placed in 100 ml of ice-cold digestion medium. This medium consisted of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.4 (referred to hereafter as MSH medium), plus 1 mM EGTA, 250 units/ml heparin, 1 mM ATP and 0.2 mg/ml Sigma protease (subtilisin BPN').

The muscle mince was digested in this medium for 30 min, 0° C. with continuous stirring. This was followed by gentle homogenization with a loose-fitting motor-driven homogenizer (Duall #24) having a ground-glass tube and pestle. The resulting homogenate was diluted to 200 ml with MSH medium plus 1 mM EGTA, 250 units/ml heparin and subjected to differential centrifugation in a Sorvall RC-2B refrigerated centrifuge, using an HB-4 swinging bucket rotor.

First, the homogenate was centrifuged for 5 min at 650 x g, and the resulting supernatant filtered through two layers of gauze and centrifuged for 15 min at 13,000 x g. The pellets from this step were resuspended in a small amount of MSH medium plus EGTA and heparin using a hand-held glass homogenizer with a teflon pestle. The suspension was diluted to about 50 ml and centrifuged at 650 x g for 5 min. The supernatant from this step, containing the mitochondria, was recentrifuged for another 5 min at 650 x g. The pellets from these two low speed centrifugations contained a light, fluffy material that was discarded. The supernatant was then centrifuged for 15 min at 13,000 x g to sediment the mitochondria. To remove traces of heparin and EGTA, the mitochondria were resuspended in MSH medium (without heparin and EGTA), diluted to 100 ml and centrifuged again for 15 min at 13,000 x g. The final pellet was resuspended in MSH medium to a volume of 2.0 ml and 25 µl samples were taken for protein estimation. Volumes were adjusted to give a final protein concentration of 5 mg/ml. Electron microscopy showed the preparations to be free of contaminants such as sarcoplasmic reticulum.

Any deviations from this standard procedure wil be mentioned when each individual experiment is described.

2. Isolation of Brown Adipose Tissue Mitochondria

Brown adipose tissue mitochondria were isolated by the procedure of Grav et al. (1970) except that the isolation medium was buffered with 1 mM

imidazole rather than HEPES.

Interscapular brown adipose tissue was excised, cleaned of white fat and skeletal muscle, weighed and chopped finely with scissors. The mince was placed in 10 ml of cold isolation medium (0.33 M sucrose, 1 mM imidazole pH 7.4), and gently homogenized with a loose-fitting all-glass homogenizer. Centrifugations were performed in the Sorvall RC-2B refrigerated centrifuge using the SS-34 rotor. The homogenate was centrifuged for 5 min at 900 x g and the supernatant filtered through 2 layers of gauze. The supernatant was then centrifuged for 5 min at 600 x g and filtered as before. The mitochondrial fraction was sedimented at 7,400 x g for 5 min. The walls of the centrifuge tube were carefully wiped with a tissue to remove adhering fat, and the mitochondrial pellet was resuspended in a small amount of isolation medium by gentle stirring with a glass rod. After dilution to 10 ml with additional medium, the mitochondria were sedimented again at 7,400 x g for 5 min. The pellet was resuspended as before, made up to exactly 10 ml, and 0.5 ml samples were withdrawn for protein estimation. The mitochondria were sedimented a final time and the pellet resuspended in 0.33 M sucrose - 1 mM imidazole to a concentration of 1 mg/ml.

3. Protein Estimation

Protein was determined by the method of Lowry et al. (1951), as modified by Schacterle and Pollack (1973). Bovine serum albumin was used as standard. Because a number of substances, including sucrose, Tris, EDTA and HEPES have been found to interfere with the Lowry protein determination (eg. Peters & Fouts, 1969; Ji, 1973), mitochondrial protein was routinely precipitated from samples of the isolation media with cold 10% (w/v, final concentration) trichloroacetic acid. After standing on ice for 30 min, the precipitated

protein was sedimented at 16,000 x g for 10 min and the pellet dissolved in 1.0 ml warm 0.5 N NaOH. The protein estimation was then carried out as described in the literature (Schacterle and Pollack, 1973).

4. Measurement of Calcium Contents of Mitochondria

The calcium content of mitochondrial fractions was determined essentially as described by Carafoli and Lehninger (1971).* Mitochondrial pellets (5 mg protein) were suspended in 0.8 ml of 0.5 N HCl and placed in a boiling water bath for 10 min. After centrifugation (10 min at 10,000 x g) the supernatants were withdrawn and the pellets extracted a second time as before. The supernatants containing the extracted calcium were combined and the volume made up to 2.0 ml with additional 0.5 N HCl. The calcium concentration was determined using a Jarrell-Ash Model 740 Dial-Atom II atomic absorption spectrophotometer. All samples and calcium standards contained 1% (w/v) $LaCl_3$ to suppress interference by phosphates (Willis, 1961), and all had identical acid concentrations of 0.5 N HCl. Calcium standards were prepared from a commercial stock calcium solution (1000 ppm, Fisher Scientific Co.).

5. Measurement of Mitochondrial Respiration

All measurements of mitochondrial oxygen uptake were determined polarographically using a Model 53 Biological Oxygen Monitor (Yellow Springs Instruments Co.) consisting of an oxygen-sensitive electrode, a constant temperature bath-stirrer assembly, and an oxygen meter connected to a strip-chart recorder.

For the determination of the effects of calcium addition on mitochondrial respiration (Results and Discussion, Part B), the incubation medium consisted of 80 mM KCl, 10 mM Tris-HCl, pH 7.4, 0.5 mM potassium phosphate, 0.2% bovine serum albumin (fatty acid free), and one of the following substrates; 10 mM

* Similar results were obtained when mitochondria were prepared for atomic absorption spectrophotometry by dry ashing - see Appendix A.

sodium α-glycerophosphate, 10 mM sodium succinate + 0.125 μM rotenone, and 5 mM sodium pyruvate + 5 mM sodium malate. Mitochondrial protein was present in concentrations of 1-1.5 mg/ml. The total volume of the incubation medium was 2 ml and the temperature was 37°C. Mitochondria were allowed to respire in this medium for 3-5 min before the addition of 25 μl of 20 mM $CaCl_2$ (Final concentration, 250 μM).

The following respiration rates were determined: the state 4 respiration rate before addition of calcium, the calcium-stimulated respiration rate, and the state 4 rate after accumulation of calcium. For these calculations the oxygen content of the medium was taken as 0.398 μg-atoms O/ml at 37°C (Chappell, 1964). From these results the respiratory control ratios (RCR's) and Ca/O ratios were determined for each substrate. The RCR is defined as the ratio of the calcium-stimulated respiration rate to the state 4 respiration rate after calcium accumulation. The Ca/O ratio is defined as the ratio of calcium added to the total oxygen consumed during calcium-stimulated respiration.

For experiments designed to test the effects of ruthenium red and ionophore A23187 on mitochondrial respiration rate (Results and Discussion, Part C) the incubation medium consisted of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.4, 0.2% bovine serum albumin, 5 mM Tris-glutamate + 5 mM Tris-malate, 1 mM potassium phosphate, and mitochondrial protein, 1 mg/ml. Ruthenium red and A23187 were added to give final concentrations of 1 and 2 n mole/mg mitochondrial protein respectively. In some experiments, ADP was added to give a final concentration of 300 μM.

Crude ruthenium red was purified by recrystallization as described by Fletcher et al. (1961) and assayed spectrophotometrically according to Luft (1971). Stock solutions of 10 mM were stored in the refrigerator and kept

no longer than one month. The ionophore A 23187 was dissolved in absolute ethanol to provide a stock solution of 10 mM and stored in the freezer until used.

6. Measurement of Calcium Uptake and Release by Muscle Mitochondria

The techniques used for measuring calcium uptake and release by isolated mitochondria are based on the studies of Reed and Bygrave (1974a, b, 1975a, b). The major problems inherent in studies of calcium kinetics is the great rapidity of the process, requiring techniques having a high time resolution. Perhaps the best method is one described by Mela and Chance (1968) which involves the use of the calcium-sensitive indicator murexide. This method, however, requires complex, expensive equipment (a dual-wavelength spectrophotometer and stopped-flow apparatus). An alternate approach is to separate mitochondria from the incubation medium by centrifugation or microfiltration at specific time intervals following the addition of the radioisotope $^{45}Ca^{2+}$. Normally the time resolution of this technique is too slow for accurate kinetic studies. However, Reed and Bygrave overcame the limitation by using reduced temperatures to slow the reaction down and by using a special quenching solution to stop the reaction before filtration. The quenching solution consists of the specific calcium chelating agent EGTA and the noncompetitive inhibitor of mitochondrial calcium transport ruthenium red. EGTA does not penetrate the mitochondrial inner membrane and so will only bind free calcium in the medium and calcium bound non-specifically to the outer surfaces of the mitochondrion. Thus this technique has the added advantage in that it measures only calcium specifically transported by the mitochondrion and eliminates the contribution of non-specific surface binding. Because the presence of EGTA alone would cause net release of mitochondrial calcium (see Literature Review, Part C, Section 1(d)), Reed and Bygrave included

ruthenium red in the quenching solution to inhibit the release process. Because these techniques do not require expensive equipment, are convenient and show high specificity, they were adopted for the studies reported in this thesis.

(a) Measurement of the Time Course of Calcium Uptake

The incubation medium consisted of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 5 mM pyruvate, 5 mM malate, 1 mM phosphate (when present) and 1 mg/ml mitochondrial protein in a total volume of 2.0 ml. All solutions were neutralized with Tris. The temperature of the medium was 5°C. The reaction was carried out in a small plastic 2 ml beaker open to the air, and the medium was stirred continuously with a micro stirring bar. After a 5 min preincubation period to allow for temperature equilibration, the reaction was started by the addition of 20 µl of 20 mM $^{45}CaCl_2$ (~5.4 mCi/m mole) to give a final calcium concentration of 200 µM. At time intervals of 0.5, 1.0, 2.5, 5.0 7.5 10.0, 12.5, 15.0, 17.5, 20.0, and 25.0 min, 0.1 ml aliquots were withdrawn and rapidly mixed with an equal volume of ice-cold quenching solution (10 mM EGTA + 6 µM ruthenium red, Reed & Bygrave, 1975b). Mitochondria were then separated from the medium by vacuum filtration through 0.22 µm Millipore filters. The filters were washed with 5 ml of cold MSH medium. No more than a few minutes elapsed between quenching and filtration. Filters were then dried under an infrared lamp for 5 min, placed in scintillation vials, and solubilized in 7 ml of Beckman Filter-Solv. To measure total radioactive calcium in the reaction medium, a sample was withdrawn without prior filtration. This aqueous sample was solubilized in a scintillation cocktail consisting of 1.0 ml NCS, 9.0 ml toluene and 5 g/l PPO. All samples were counted in a Beckman LS-250 liquid scintillation counter, and correction for quenching was made using the channels ratio method.

(b) Measurement of Initial Rates of Calcium Uptake

Methods were the same as just described, except that samples were taken at time intervals of 10, 20, 30, 45 and 60 sec. Initial rates were determined from the slope of the graph of calcium uptake (ng-atoms/mg protein) vs. time (min); slopes were determined by linear regression analysis.

(c) Measurement of Calcium Release

Essentially the same techniques just described for uptake studies were also used to test the effects of various releasing agents on the retention of calcium by mitochondria. Because a variety of conditions were employed depending on the releasing agent studied, it is more convenient to outline here a general method and give more specific details of each individual experiment when the results are given in Chapter IV.

Mitochondria were allowed to accumulate ^{45}Ca until saturation with respect to uptake was reached, and then the particular releasing agent was added (EGTA, ruthenium red, cyclic AMP, Na^+, prostaglandin E_1). Samples were then withdrawn at timed intervals and filtered through Millipore filters, which were washed, dried and counted as before. This gives a value for the amount of calcium remaining in the mitochondrion at each time interval, from which the amount of calcium released can be determined.

7. Measurement of High Affinity and Low Affinity Binding Sites of Mitochondria

The respiration-inhibited high affinity and low affinity binding sites were studied essentially as described by Reynafarje and Lehninger (1969). Mitochondria were isolated in the usual manner but the final mitochondrial pellet was resuspended in 250 mM sucrose, 2 mM Tris-HCl, pH 7.4, to a protein concentration of 5 mg/ml.

The incubation medium was made up of 250 mM sucrose, 2 mM Tris, pH 7.4, 1 mg/ml mitochondrial protein, 0.25 μM rotenone, 0.225 μM antimycin A. The

inhibitors were added as ethanolic solutions. Concentrations of $^{45}CaCl_2$ ranged from 1.0-500 ng-atoms/mg mitochondrial protein. The total volume was 0.5 ml and the temperature, 0°C. Reactions were carried out in small 1.5 ml Eppendorf plastic micro centrifuge tubes. To 0.2 ml mitochondrial suspension, 0.1 ml of sucrose-tris containing the inhibitors was added. After 1 min, 0.2 ml of sucrose-tris containing a particular amount of $^{45}Ca^{2+}$ was added. After a 1 min incubation, tubes were centrifuged for 2 min in an Eppendorf model 3200 micro centrifuge to separate mitochondria from the medium. The radioactivity remaining in the supernatant was measured by withdrawing a 0.2 ml aliquot and solubilizing it in 1.0 ml NCS, 9 ml toluene, 5 g/l PPO. From these measurements the amount of calcium bound to the mitochondria and the calcium remaining in the medium were calculated for each concentration of calcium added.

The number and affinity of each class of binding site was determined by Scatchard analysis as outlined by Reynafarje and Lehninger (1969). A graph of bound calcium (ng-atoms/mg protein)/free calcium (μM) vs. bound calcium (ng-atoms/mg protein) yields a biphasic curve having a high affinity leg intersecting the ordinate and a low affinity leg intersecting the abscissa. By extrapolating the high affinity leg to the abscissa and the low affinity leg to the ordinate, the number (n) and stability constant (K') of each class of site can be determined. Intercepts on the abscissa give the number of sites n, while intercepts on the ordinate give nK', from which K' can be calculated. The reciprocal of K' gives K, the dissociation constant for the Ca^{2+}-binding site complex. This quantity is equivalent to the concentration of calcium at which the binding sites are half-saturated, and this is a measure of the affinity of the site for calcium.

8. Statistical Analysis of Results

Results are expressed as mean ± standard error of the mean (S.E.M.). Significance of differences was tested using the Student t test (Snedecor, 1956).

CHAPTER IV: RESULTS AND DISCUSSION

The results of each experiment will be presented in the following manner:

Section 1; Purpose of the Experiment

2: Description of the Experiment

3: Results and Discussion

PART A: CALCIUM CONTENTS OF SKELETAL MUSCLE AND BROWN ADIPOSE TISSUE MITOCHONDRIA FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

Changes in the calcium transporting properties of muscle mitochondria as a result of cold-acclimation may be reflected in a difference in the calcium content of the mitochondria. The purpose of this experiment is to isolate in various ways muscle mitochondria from warm- and cold-acclimated rats, measure calcium contents of the mitochondrial fractions, and see if any differences exist which might be related to the *in vivo* functioning of the mitochondria. For comparative purposes, calcium is also measured in brown adipose tissue mitochondria.

2. Description of the Experiment

A total of 38 rats, 19 warm-acclimated (WA) and 19 cold-acclimated (CA), had lived approximately 3 months at their respective acclimation temperatures before the start of the experiment. The mean weights of the rats were: WA: 519 ± 21 g; CA: 400 ± 14 g.

Interscapular brown adipose tissue mitochondria were isolated from 8 WA and 8 CA rats in 0.33 M sucrose, 1 mM imidazole exactly as described in the Methods. Muscle mitochondria were prepared from 8 WA and 8 CA rats by the

standard isolation procedure (see Methods), except that calcium chelating agents (EGTA, EDTA) were omitted from isolation media to avoid excessive removal of mitochondrial calcium during isolation. This presents another problem, however, because in the absence of EDTA muscle mitochondria tend to bind additional calcium during isolation because of the high calcium content of this tissue. To avoid this, Thakar, Wrogemann and Blanchaer (1973) recommended the inclusion of 2.5 μM ruthenium red in isolation media. Thus, for these experiments, the muscle from each rat was divided into two lots, and mitochondria from one lot were prepared in MSH medium, while mitochondria from the other lot were prepared in MSH medium + 2.5 μM ruthenium red.

Because the proteolytic digestion step used to isolate the mitochondria could conceivably influence the calcium contents, mitochondria were also prepared from 3 WA and 3 CA rats by a procedure that does not require proteolytic digestion. This procedure utilizes an ionic medium (100 mM KCl) rather than a sucrose medium, and a full description of the method is found in Ernster and Nordenbrand (1967). Again, one-half of the mitochondria from each rat were isolated in a medium containing ruthenium red. Calcium contents of mitochondrial pellets were determined by atomic absorption spectrophotometry as described in the Methods section.

Results and Discussion

The results of the experiment are summarized in Table II and Figure 14.

The most striking observation of this study is the large reduction in the calcium contents of IBAT mitochondria as a result of cold-acclimation. This finding agrees with that of Christiansen (1971) who observed that the calcium content of newborn guinea pig BAT mitochondria was very much lower than that

TABLE II

Calcium Contents of Muscle and IBAT Mitochondria

Experiment	WA	CA	P (W vs C)
IBAT (n=8)	44.2 ± 3.2	20.0 ± 1.3	P < 0.001
Skeletal Muscle			
A. MSH medium (n=8)			
-RR	14.5 ± 1.4	15.1 ± 1.2	NS
+RR	15.3 ± 0.8	15.4 ± 1.1	NS
P (-RR vs +RR)	NS	NS	
B. KCl medium (n=3)			
-RR	24.9 ± 0.7	22.2 ± 2.1	NS
+RR	16.6 ± 0.8	16.6 ± 1.0	NS
P (-RR vs +RR)	P < 0.005	NS	

Results are given as mean ± S.E. Units are ng-atoms calcium/mg protein.

Abbreviations: WA: warm-acclimated; CA: cold-acclimated; IBAT: interscapular brown adipose tissue; MSH: 210 mM mannitol, 70 mM sucrose, 10 mM HEPES; RR: ruthenium red.

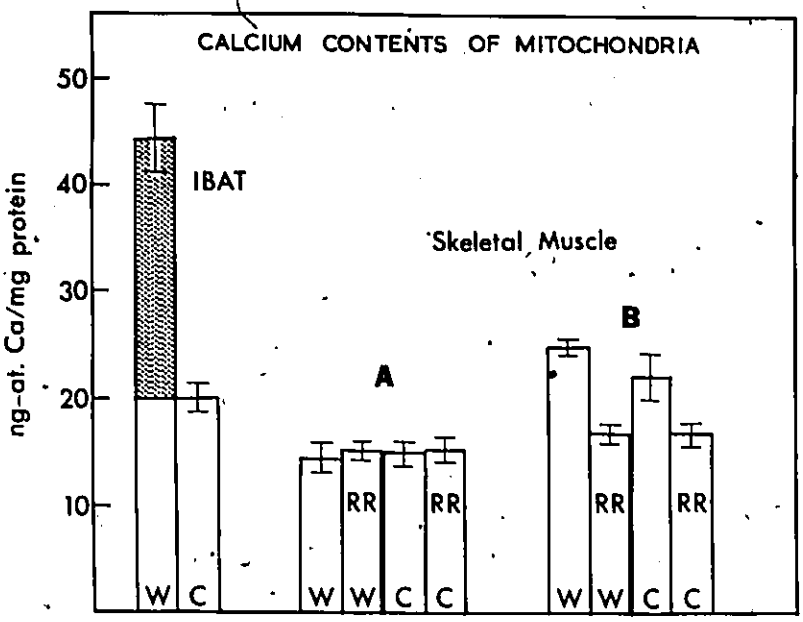

Figure 14. Calcium Contents of Isolated Brown Adipose Tissue and Skeletal Muscle Mitochondria from Warm- and Cold-Acclimated Rats

The graph compares the calcium contents (ng-atoms Ca/mg mitochondrial protein) of interscapular brown adipose tissue (IBAT) and skeletal muscle mitochondria isolated from warm-acclimated (W) and cold-acclimated (C) rats. IBAT mitochondria were isolated in 0.33 M sucrose, 1 mM imidazole pH 7.4. Skeletal muscle mitochondria were isolated by two methods: A: MSH medium and B: 100 mM KCl medium (no proteolytic digestion). The bars designated RR signify calcium contents of mitochondria isolated in the presence of 2.5 μM ruthenium red. Shaded areas of bars refer to a significant difference in calcium content between mitochondria from a warm-acclimated and cold-acclimated rat. Values are shown as mean ± S.E. (vertical lines).

of weaned guinea pigs. In other words, transformation of BAT from a non-thermogenic to a thermogenic state (newborn or cold-acclimated animal) gives rise to a reduction in mitochondrial calcium levels. Whether this actually happens *in vivo* is uncertain. Both thermogenic and nonthermogenic BAT mitochondria would be in an uncoupled state as a result of this isolation procedure, and so a certain amount of the mitochondrial calcium would be expected to leak out during isolation. It is quite possible that the mitochondrial membrane of thermogenic BAT is more permeable to ions than nonthermogenic BAT, and so a greater proportion of calcium is lost. Christiansen (1971) postulated that the mitochondrial calcium pool is more mobile when BAT is in a thermogenic state. In any event, these possibilities were not explored further since brown adipose tissue is not the major subject of the research reported here.

Unlike BAT mitochondria, no difference between CA and WA rats were found for skeletal muscle mitochondria whether isolated in the MSH medium after proteolytic digestion or in the KCl medium without digestion. The inclusion of ruthenium red had no effect on the calcium levels in mitochondria isolated in MSH medium. This agrees with the findings of Thakar *et al.* (1973), who isolated mitochondria from hamster muscle by a very similar procedure to the one used here. They found that ruthenium red could prevent binding of calcium during isolation but the effects were only noticeable if a large amount of additional calcium was added to the homogenate. No difference was seen in the absence of exogenous calcium. In other words, uptake of endogenous cell calcium by muscle mitochondria is not a problem with this particular isolation procedure. On the other hand, calcium levels of mitochondria prepared in the KCl medium were slightly higher and the presence of ruthenium red lowered this level to that seen using the MSH medium. It appears, then,

that mitochondria did bind additional calcium when isolated in the KCl medium.

The calcium contents of muscle mitochondria observed in this study are in the same range as values found for mitochondria from other tissues. Rat liver and rat heart mitochondria, for example, contain approximately 12 and 30 ng-atoms Ca^{2+}/mg protein respectively (Carafoli & Lehninger, 1971). Hamster skeletal muscle mitochondria contain about 22 ng-atoms Ca^{2+}/mg protein (Thakar et al., 1973). Interestingly, muscle mitochondria from dystrophic hamsters have greatly elevated calcium contents, in excess of 200 ng-atoms/mg protein, and as a result a defect in oxidative phosphorylation exists (Wrogemann, Blanchaer & Jacobson, 1970). Apparently only a small fraction of the mitochondrial population has this defect, the remainder being normal and having the usual calcium content (Mezon, Wrogemann & Blanchaer, 1974).

The finding that cold-acclimation does not give rise to changes in the muscle mitochondrial calcium contents would rule out any suggestion of a mechanism of heat production in which mitochondria become uncoupled due to the uptake of damaging amounts of calcium, as is postulated in malignant hyperthermia. (Literature Review, Part B, page 34). It does not, however, rule out a mechanism involving enhanced recycling of mitochondrial calcium. Here, the steady-state level of mitochondrial calcium would be the same in the WA rat and the CA rat; only the rates of uptake and efflux would be changed.

Bramante and Nirdlinger (1974) measured the subcellular ionic distribution of ions in liver and heart of cold-acclimated rats. They found that calcium contents of heart mitochondria were unchanged while those of liver mitochondria were slightly reduced compared to controls (6.2 vs. 4.7 ng-atoms/mg protein). Although this difference was statistically significant ($P<0.05$), it is quite small and its meaning is not readily apparent. McBurney and Radomsky (1973)

observed a similar reduction in the calcium contents of liver mitochondria in CA rats, but this was only seen if EDTA was included in the isolation medium. Both groups of authors suggested that the reduction in calcium levels was due to an uncoupling of liver mitchondria as a result of cold-acclimation. However, it is generally accepted today that uncoupling of liver mitochondria is not involved in nonshivering thermogenesis. Because liver mitochondria are more fragile in CA rats (Lusena & Depocas, 1967), a possible explanation for the findings of these workers is that a greater percentage of the liver mitochondria from the CA rat become damaged during homogenization, allowing a greater amount of calcium to leak out during isolation. This would give rise to an apparent reduction in the calcium content of the mitochondrial fraction.

In all of these studies one must be careful not to draw too many conclusions from calcium determinations on isolated mitochondria. Even though an attempt was made to minimize shifts in calcium distribution during homogenization of the muscle by excluding calcium chelating agents and by adding ruthenium red, it is uncertain whether the values obtained reflect the true in vivo calcium contents of the mitochondria.

PART B: THE EFFECTS OF CALCIUM ON RESPIRATION OF SKELETAL MUSCLE MITOCHONDRIA FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

Muscle mitochondria from cold-acclimated rats were found previously to show increased state 3 respiration rates and somewhat smaller increases in state 4 respiration rates with a variety of substrates (Himms-Hagen et al., 1975; Hbous, 1976). It is of interest then, to see whether calcium-stimulated respiration rates (analagous to state 3 or ADP stimulated rates) are also

elevated as a result of cold-acclimation. In this experiment, calcium-stimulated respiration as well as respiratory control ratios and Ca/O ratios are compared for muscle mitochondria from WA and CA rats. Changes in the two latter parameters might indicate calcium-associated alterations in mitochondrial control mechanisms.

2. Description of the Experiment

Ten warm-acclimated rats weighing 500 ± 16 g and 10 cold-acclimated rats (380 ± 19 g, acclimated about 3 months) were selected for these experiments. Preliminary work established the ideal incubation medium for obtaining optimal respiratory control ratios and Ca/O ratios. A description of this medium and the procedure followed may be found in the Methods.

3. Results and Discussion

The values obtained for calcium-stimulated respiration rates, state 4 rates before and after calcium addition, RCR's and Ca/O ratios for the substrates α-glycerophosphate, succinate and pyruvate-malate are summarized in Table III and illustrated graphically in Figure 15.

First, it can be seen that the state 4 rates before calcium addition were slightly elevated in mitochondria from the CA rat (although the increase with pyruvate-malate as substrate was not statistically significant). Calcium-stimulated rates were significantly higher in mitochondria from CA rats with α-glycerophosphate and pyruvate-malate as substrates, but not with succinate. Finally, the state 4 rates measured after the accumulation of calcium were significantly increased with all three substrates. No differences between WA and CA rats were observed in either RCR's or Ca/O ratios.

These changes should be compared with the changes in ADP-stimulated respiration in muscle mitochondria of CA rats (Figure 12, page 45). Keeping

TABLE III

The Effects of Calcium on Respiration of Skeletal Muscle Mitochondria from Warm- and Cold-Acclimated Rats

	α-glycerophosphate			succinate			pyruvate + malate		
	WA	CA	P	WA	CA	P	WA	CA	P
State 4 (before Ca^{2+} addition)	72.2 ± 2.7	83.9 ± 2.5	0.005	121.2 ± 4.0	134.9 ± 4.4	0.05	47.0 ± 1.8	52.0 ± 1.7	NS
Ca^{2+}-stimulated	169.7 ± 7.0	206.5 ± 9.2	0.01	359.2 ± 21.8	359.1 ± 25.5	NS	172.9 ± 6.5	197.0 ± 8.7	0.05
State 4 (after Ca^{2+} accumulation)	81.8 ± 4.2	104.4 ± 5.7	0.005	117.0 ± 5.8	136.9 ± 6.2	0.05	47.2 ± 1.8	53.5 ± 1.7	0.02
RCR	2.09 ± 0.08	1.99 ± 0.05	NS	2.99 ± 0.16	2.62 ± 0.13	NS	3.67 ± 0.08	3.70 ± 0.13	NS
Ca/O	3.14 ± 0.20	3.34 ± 0.16	NS	3.50 ± 0.09	3.65 ± 0.15	NS	5.99 ± 0.19	6.18 ± 0.20	NS

The rates are expressed as mean ± S.E.; units are ng-atoms O/min/mg protein, at 37° C.

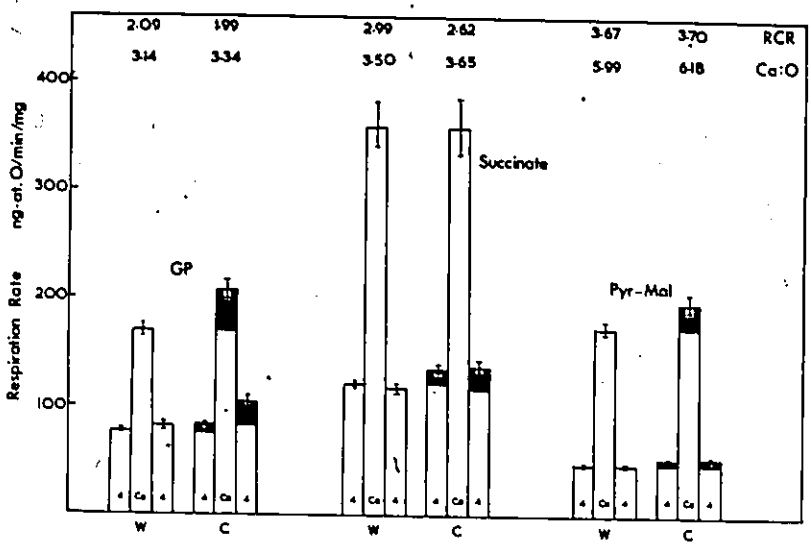

Figure 15. The Effects of Calcium on Respiration of Skeletal Muscle Mitochondria from Warm- and Cold-Acclimated Rats

This graph illustrates and compares the respiration rate of muscle mitochondria isolated from warm-acclimated (W) and cold-acclimated (C) rats using three different respiratory substrates: α-glycerophosphate (GP), succinate, and pyruvate-malate (Pyr-Mal). Each bar signifies from left to right, state 4 respiration rate before Ca^{2+} addition (4), the calcium-stimulated rate (Ca), and the state 4 rate after Ca^{2+} uptake (4). Shaded portions of the bars signify the increases in respiration rate found in mitochondria from cold-acclimated rats. Vertical lines at the top of each bar represent standard errors of the mean. Also shown at the top of the graph are respiratory control ratios (RCR) and Ca/O ratios for each substrate. The temperature of the experiment was 37^{o} C.

in mind that a different incubation medium was used in these experiments, one can see that the increase in response of muscle mitochondria from CA rats to added calcium is qualitatively similar to the increase in response to ADP in the same mitochondria although the latter was larger. With α-glycerophosphate as substrate the increase in calcium-stimulated respiration was 22% whereas the increase in ADP-stimualted respiration was 60%. For pyruvate-malate the percent increases were 14% (calcium) and 32% (ADP), and for succinate, 0% (calcium) and 15% (ADP). State 4 respiration rates were slightly higher in mitochondria from CA rats both after Ca^{2+} accumulation and after ADP phosphorylation. In muscle mitochondria from CA rats, no changes take place in ADP/O or Ca/O ratios or in the respective RCR's.

The reason that no increase was found in the calcium-stimulated rate with succinate as substrate is not readily apparent. Rates were always very high with this substrate and quite variable, as indicated by the large standard errors of the mean (Table III). It is possible that the precision of the measurements are not sufficiently high to detect a difference. The percent increase found in calcium-stimulated respiration rates with α-glycerophosphate and pyruvate-malate as substrates were a little less than half the corresponding increases in ADP-stimulated rates. By analogy, then, one would expect the calcium-stimulated rate for succinate to be only about 5%, which might not be detectable by the techniques used here.

The fact that no change was found in RCR's or Ca/O ratios indicates that the changes the muscle mitochondria undergo during cold-acclimation are not like those of the mitochondria of brown adipose tissue. Christiansen (1971) studied the effects of calcium on respiration of BAT mitochondria from newborn, cold-stressed and weaned guinea pigs and found that thermogenic BAT mitochondria

required the presence of nucleotides to show respiratory control with calcium. A similar nucleotide requirement is seen for respiratory control with ADP (see Literature Review, page 36). The unchanged RCR and Ca/O ratios reported here were expected in light of previous findings in this laboratory that muscle mitochondria do not become uncoupled or loosely-coupled as a result of cold-acclimation as evidenced by normal RCR's and ADP/O ratios (Himms-Hagen et al., 1975; Hbous, 1976); the only differences found in the muscle mitochondria from CA rats were increases in ADP-stimulated respiration rates and smaller increases in state 4 rates (after ADP). These observations may now be extended to calcium-stimulated respiration rates.

A likely explanation for increased ADP-stimulated respiration rates is the finding that the activity of the ADP translocase is elevated by 35% in the CA rat (Hbous, 1976). Thus ADP is made available to the coupling site of the respiratory chain at a greater rate in mitochondria from the CA rat. One could postulate, then, that a similar mechanism exists for calcium. In the CA rat the activity of the calcium transport system could be enhanced, thereby allowing a greater rate of calcium transport into the mitochondrion. This could result from either an increase in the affinity of the carrier for calcium or an increased number of calcium carriers per mitochondrion. Experimental investigation of these possibilities will be presented later in the thesis.

It should also be pointed out that at least part of the increase in calcium-stimulated respiration rate seen in mitochondria from CA rats with α-glycerophosphate as substrate may be due to the fact that the activity of α-glycerophosphate dehydrogenase is 60% higher in these mitochondria (Hbous, 1976). This enzyme is also activated by extremely low levels of calcium

(0.1 μM) (Carafoli & Sacktor, 1972). It is also possible that the addition of calcium activates the enzyme to a greater extent in mitochondria from CA rats in these experiments.

Another interesting aspect of these studies and the earlier work of Hbous (1976) is the increased state 4 respiration rate (after ADP-stimulation and after calcium-stimulation) seen in muscle mitochondria from CA rats. Energy dissipation during state 4 respiration results from the maintenance of various gradients across the mitochondrial inner membrane. These include the maintenance of an asymmetric distribution of ATP and ADP (Stucki & Walter, 1972), recycling of endogenous calcium (Stucki & Ineichen, 1974) and the recycling of protons (Stucki, 1975). Conceivably, any of these processes could be working at a higher level in muscle mitochondria from the CA rat. The next part of the thesis looks at the recycling of endogenous calcium as a possible mechanism for increasing state 4 rates.

PART C: THE EFFECTS OF RUTHENIUM RED AND IONOPHORE A23187 ON RESPIRATION OF MUSCLE MITOCHONDRIA FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

The purpose of this experiment is to test the effects of ruthenium red and A23187 on state 4 respiration rates of isolated muscle mitochondria from WA and CA rats. Ruthenium red has been used by Stucki and Ineichen (1974) to inhibit that portion of state 4 respiration of liver mitochondria due to recycling of endogenous calcium. Similar experiments with muscle mitochondria should tell if calcium recycling contributes more to state 4 respiration in the CA rat and if it is responsible for the increased state 4 rates seen in these mitochondria.

The divalent cation ionophore A23187 causes the efflux of calcium and magnesium from mitochondria and stimulates respiration because the released calcium is reaccumulated (Reed & Lardy, 1972; Wong et al., 1973). A second purpose of the experiments reported in this section is to see whether the fast calcium recycling induced by the ionophore occurs at a greater rate in muscle mitochondria from the CA rat.

2. Description of the Experiment

Five WA rats weighing 477 ± 15 g and 5 CA rats weighing 404 ± 14 g, acclimated 3-4 months, were selected for these experiments. Respiration was measured using the techniques outlined in the Methods, in an incubation medium consisting of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, pH 7.4, 0.2% bovine serum albumin, 5 mM Tris-glutamate, 5 mM tris-malate, 1 mM potassium phosphate and mitochondrial protein, 1 mg/ml. The effects of ruthenium red were tested on state 4 respiration rates measured both before and after ADP-stimulated state 3 rates. Ionophore A23187 was added to mitochondria respiring in state 4 both in the presence and absence of inorganic phosphate.

3. Results and Discussion

(a) Effects of Ruthenium Red

The concentration of ruthenium red required to produce an inhibitory effect on mitochondrial respiration was found to be critical in these experiments. The maximal inhibition of state 4 respiration rate occurred at 1 n mole ruthenium red/mg mitochondrial protein. Concentrations below and above this value inhibited the rate to a lesser extent, but above 5 n mole/mg protein respiration rate was enhanced, possibly because of uncoupling effects induced by the dye at these high concentrations.

Table IV summarizes the results of experiments in which 1 n mole ruthenium

TABLE IV

The Effects of Ruthenium Red and A23187 on Respiration of Muscle Mitochondria from Warm- and Cold-Acclimated Rats

		WA	CA	P<
A.	Effects of Ruthenium Red			
(a)	state 4 rate (before ADP)	61.3 ± 1.0	67.2 ± 1.7	0.02
	RR present	53.1 ± 1.3	57.4 ± 2.0	NS
	% inhibition	15%	17%	
(b)	state 4 rate (after ADP)	75.8 ± 6.4	86.7 ± 7.3	NS
	RR present	60.0 ± 4.0	68.3 ± 3.0	NS
	% inhibition	26%	26%	
B.	Effects of A23187			
(a)	state 4 rate (+Pi)	61.0 ± 1.7	69.4 ± 3.0	0.05
	A23187 present	93.1 ± 4.0	104.3 ± 5.6	NS
	% activation	52%	50%	
(b)	state 4 rate (-Pi)	36.4 ± 1.3	40.5 ± 1.2	0.05
	A23187 present	107.3 ± 4.4	114.1 ± 2.7	NS
	% activation	194%	181%	

The rates are expressed as mean ± S.E.M.; units are ng-atoms O/min/mg. at 37° C.

red/mg protein was added to mitochondria either before or after ADP addition. Ruthenium red caused an approximately equal inhibition of state 4 respiration rates in mitochondria from WA and CA rats, whether added before ADP or after ADP-stimulated respiration had ceased. This implies that the proportion of state 4 respiration due to endogenous calcium recycling is no different in the CA rat. Unfortunately, with the substrate used in these experiments (glutamate-malate), the state 4 rates (after ADP) were quite variable and the increase seen in mitochondria from CA rats was not statistically significant (Table IV). No significant increase was found either in the experiments of Hbous (1976, Figure 12) in state 4 rates (after ADP) with glutamate as substrate. In retrospect, it would have been better to use pyruvate-malate because the increase seen in state 4 rates with this substrate is relatively large (see Figures 12 and 15). One might then have been able to see if the increase was due to enhanced calcium recycling.

However, the validity of using ruthenium red to measure calcium recycling is in question in light of the studies reported here. Because the inhibition of the state 4 rate was dependent on the concentration of ruthenium red added, one has to wonder whether this compound is specifically inhibiting the calcium recycling process alone. As discussed in the Literature Review (page 57), different workers have noted different effects of ruthenium red towards calcium uptake and release. Because this compound binds nonspecifically to mucopolysaccharides, it seems reasonable to believe that it would cause changes in membrane properties and could influence mitochondrial processes other than calcium transport.

(b) Effects of A23187

The effects of this ionophore on state 4 respiration rate are summarized in Table IV. In the presence and absence of phosphate, A23187 stimulated

respiration but the increases observed were approximately the same in mitochondria from WA and CA rats. Note that state 4 respiration rates were lower in the absence of phosphate and so the percent increase in rate induced by the ionophore are much greater than in the presence of phosphate. According to Reed and Lardy (1972) and Wong et al. (1973), the reason for the A23187-induced stimulation of oxygen uptake by mitochondria is the release of mitochondrial calcium followed by its immediate energy-linked reuptake. This conclusion was based on observations that the rate increase could be blocked by La^{3+} or ruthenium red which inhibit the calcium carrier, or by EGTA and EDTA, which chelate released calcium and prevent its reuptake.

The fact that recycling induced by the ionophore appears to be no different in mitochondria from CA rats compared to controls implies that there is no increase in the activity of the calcium transport system in muscle mitochondria as a result of cold-acclimation. This finding does not eliminate a heat-producing mechanism involving calcium recycling because A23187 is not a naturally-occurring substance in mammalian systems. Moreover, the cycling of calcium induced by A23187 is dependent on the calcium available in the mitochondria, shown already to be the same in CA and WA rats. It is still possible that muscle mitochondria from the CA rat show a greater sensitivity to releasing agents such as cyclic AMP and prostaglandins. The fact that calcium-stimulated respiration rates were elevated, albeit to a small extent in the CA rat, suggests that some difference exists in the calcium transporting ability of these mitochondria. To test this implication further, the best approach would be to monitor the uptake and release of calcium directly rather than by using oxygen uptake as an indicator of calcium accumulation. Results of such experiments are reported in the next two parts of the thesis.

PART D: MEASUREMENT OF THE UPTAKE OF CALCIUM BY MUSCLE MITOCHONDRIA FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

A mechanism of nonshivering thermogenesis in muscle involving calcium recycling might involve an adaptive change in the calcium transport system which would enable the mitochondria to accumulate calcium at a greater rate or to a greater extent than normal. This idea is supported by the results of the previous experiment that showed that muscle mitochondria from the CA rat have enhanced calcium-stimulated respiration rates. In the experiments reported in this part, initial rates of calcium uptake and the time course of calcium uptake are compared in muscle mitochondria from WA and CA rats. In addition, initial rates of calcium uptake are measured in rats undergoing acclimation to cold to see whether this process undergoes changes during the development of NST.

2. Description of the Experiment

For measurement of the initial rates and time course of calcium uptake, 6 warm-acclimated rats weighing 467 ± 21 g and 6 cold-acclimated rats weighing 376 ± 12 g were used. These animals had lived either in the cold or the warm for about 3 months. For the experiments in which the initial rate of calcium uptake was followed during acclimation to cold, a large group of young rats recently acquired from the breeder and weighing 150-200 g was divided into two groups and placed either in the cold room or at room temperature. Measurements of the initial rate of calcium uptake of the muscle mitochondria were made on 3 cold-exposed and 3 control rats after each of the following number of days in either the cold or the warm: 1, 7, 16, 23 and 37. The procedures used for determining calcium uptake are described in detail in the Methods.

3. Results and Discussion

Since the techniques used here to measure calcium uptake were originally developed for liver mitochondria (Reed & Bygrave, 1975b), certain preliminary experiments were carried out to make sure they could be successfully applied to the study of muscle mitochondria.

As outlined in the methods (page 86), calcium uptake at various time intervals is quenched by the addition of EGTA + ruthenium red. It is important to verify that these compounds actually do prevent further uptake of calcium and that they do not themselves induce efflux of accumulated calcium. Figure 16 illustrates the retention of calcium by mitochondria after the uptake was stopped by the EGTA-ruthenium red quench solution. In both the presence and absence of phosphate, the muscle mitochondria from either WA or CA rats did not accumulate any additional calcium. In the absence of phosphate, no calcium was lost by the mitochondria even after 10 min in the quench solution. With phosphate present, a small amount of calcium did leak out after this time period. However, mitochondria were always separated from the medium by filtration within a few minutes after quenching. After this time the loss of calcium even in the presence of phosphate was negligible. Similar results were found when the uptake was quenched at various times after calcium addition. Thus the EGTA-ruthenium red quench method developed by Bygrave for measuring calcium uptake is suitable for use with muscle mitochondria.

In any enzyme kinetic study one must establish that the rate of the reaction is proportional to the enzyme concentration. Figure 17 shows the effects of varying the mitochondrial protein concentration on the initial rate of calcium uptake. The reaction was clearly still linear at the protein concentration of 1 mg/ml recommended by Reed and Bygrave (1975b) and routinely used in the studies reported here.

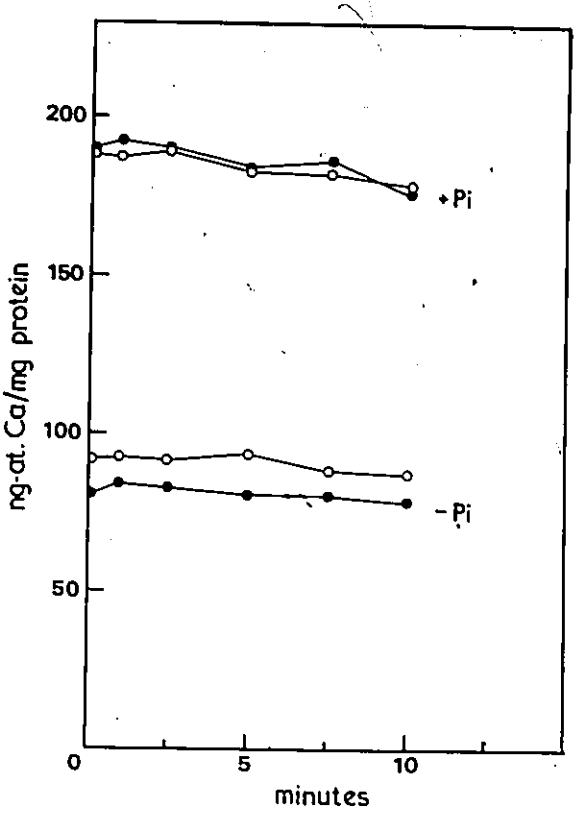

Figure 16. Effects of the EGTA-Ruthenium Red Quench Solution on the Retention of Accumulated Calcium by Muscle Mitochondria

Muscle mitochondria isolated from warm-acclimated (•) and cold-acclimated (o) rats were allowed to accumulate ^{45}Ca (total concentration, 200 µM) for 15 min in an incubation medium consisting of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.4, 5 mM pyruvate, 5 mM malate, 1 mg/ml mitochondrial protein, ± 1 mM phosphate (Pi) at a temperature of 5°C. At time 0, the reaction was quenched by the addition of an equal volume of EGTA-ruthenium red quench solution (10 mM EGTA, 6 µM ruthenium red dissolved in MSH medium). Samples were withdrawn at the times indicated on the graph, filtered, and the calcium content remaining in the mitochondria determined by measuring the radioactivity of the filters. Each point is the mean of experiments using 3 individual rats.

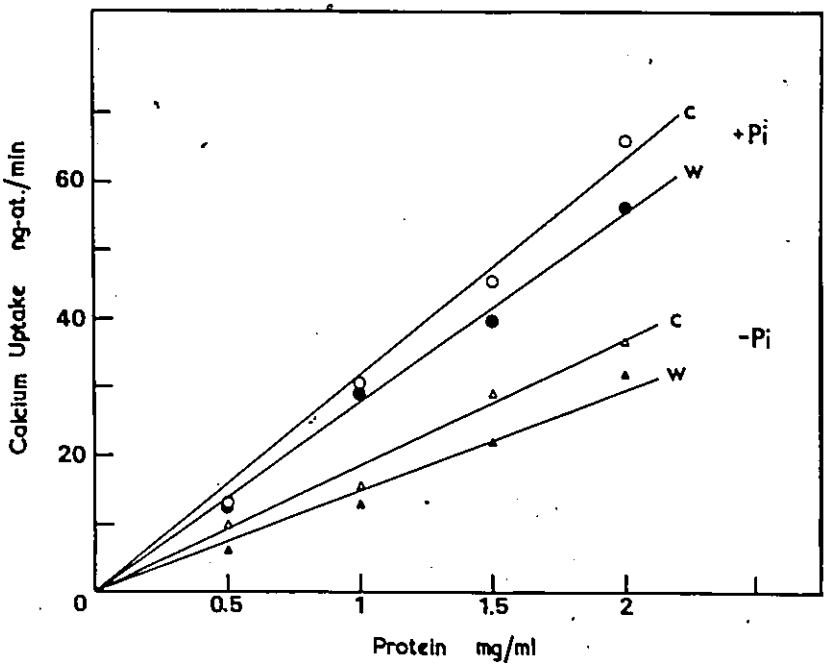

Figure 17. The Effects of Mitochondrial Protein Concentration on the Initial Rate of Calcium Uptake by Muscle Mitochondria

The graph shows the effects of varying the mitochondrial protein concentration on the initial rate of calcium uptake by muscle mitochondria from warm-acclimated (W, closed symbols) and cold-acclimated (C, open symbols) rats. The incubation conditions were as described in Fig. 16. Measurements were performed either in the presence (circles) or absence (triangles) of 1 mM phosphate (Pi). Temperature was 5°C. Each point represents the mean of experiments using either 3 (-Pi) or 4 (+Pi) individual rats.

Phosphate, a permeant anion for calcium accumulation by mitochondria, would be expected to stimulate the initial rate of calcium uptake. This is indeed the case as shown in Figure 18. The phosphate concentration chosen for routine use in the experiments in which the initial rates and time course of calcium uptake were compared in mitochondria from WA and CA rats was 1 mM, even though a greater initial rate of uptake was seen at higher phosphate concentrations. The reason for this choice is that phosphate concentrations much above 1 mM were found to have adverse effects on the retention of calcium by mitochondria. For example, muscle mitochondria from both WA and CA rats lost accumulated calcium rapidly in the presence of 5 mM phosphate, but retained it for long periods of time if the phosphate concentration was reduced to 1 mM. Other workers have noted that excessive phosphate abolishes the stoichiometric relationship between calcium accumulation and oxygen uptake (Rossi & Lehninger, 1964; Thorne & Bygrave, 1973).

(a) The Time Course of Calcium Uptake

Figure 19 illustrates the time course of calcium uptake by muscle mitochondria from WA and CA rats, both in the presence and absence of 1 mM phosphate. Looking first at the results obtained with phosphate present, one can see that mitochondria from both groups of rats accumulated virtually all of the calcium added to the medium (200 ng-atoms/mg protein) by 10 min after addition. This is a matrix loading situation, since the presence of the permeant anion allows the uptake of a large amount of calcium by virtue of its transport into the mitochondrial matrix and its deposition there as calcium phosphate salts. If the first two minutes of the time course is examined, however, it is clear that mitochondria from the CA rats accumulate calcium at a greater rate than those from the WA rats (see below, Part (b)). Uptake is

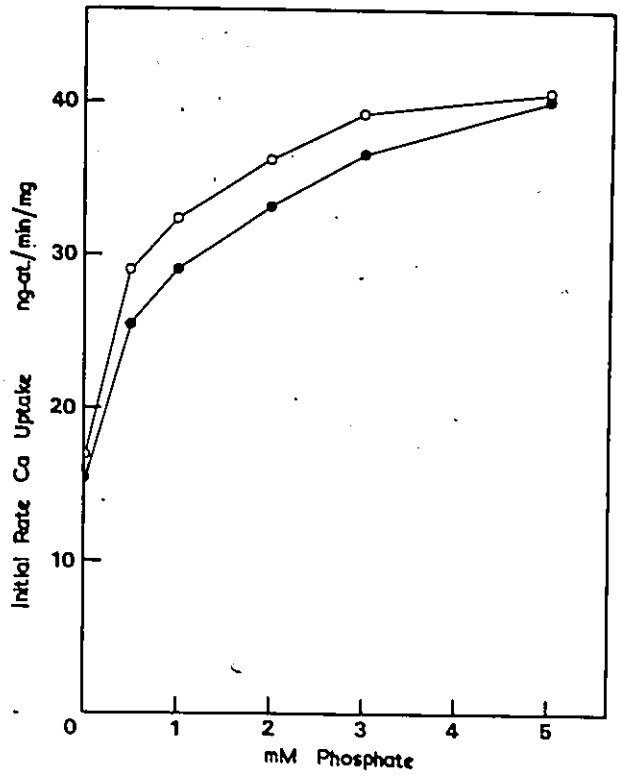

Figure 18. The Effect of Phosphate Concentration on the Initial Rate of Calcium Uptake by Muscle Mitochondria

The initial rate of calcium uptake was measured on mitochondria from a warm-acclimated rat (•) and a cold-acclimated rat (o) as a function of phosphate concentration. The incubation conditions were as described in Fig. 16. Phosphate was present at the indicated concentrations. Temperature was 5°C and the reaction was started by the addition of $^{45}CaCl_2$ (final concentration, 200 μM).

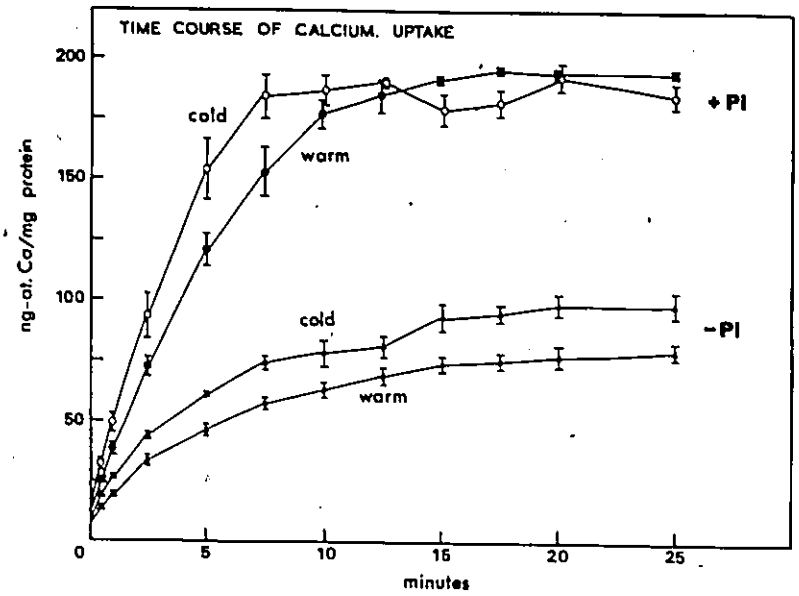

Figure 19. The Time Course of Calcium Uptake by Muscle Mitochondria from Warm- and Cold-Acclimated Rats in the Presence and Absence of Phosphate

The graph shows the time course of calcium uptake by muscle mitochondria from warm-acclimated (closed symbols) and cold-acclimated rats (open symbols). The incubation conditions were as described in Fig. 16. ^{45}Ca was added to start the reaction at a final concentration of 200 µM. The two upper curves (circles) represent uptake in the presence of 1 mM phosphate, while the two lower curves show uptake in the absence of phosphate. The reaction was stopped at appropriate time intervals by withdrawing of an aliquot of the reaction medium and placing it in an equal volume of EGTA-ruthenium red quench solution. This was followed by filtration and counting of the radioactivity remaining on the filters. The values shown are the means of experiments using 5 individual rats. Standard errors are represented by the vertical lines at each point on the graph. Temperature was 5° C.

complete by about 7 min (CA rats) compared to 10 min for WA rats.

The two lower curves illustrate calcium uptake under membrane loading conditions, that is, in the absence of the permeant anion phosphate. It will be recalled that under these conditions, mitochondria have only a limited capacity for calcium uptake, less than 100 ng-atoms/mg protein. Comparing uptake by muscle mitochondria from WA and CA rats, the graph shows that mitochondria from the CA rat not only accumulate calcium at a greater rate, but uptake reaches saturation at a higher level as well. Under the conditions of the experiment, the capacity of muscle mitochondria from WA rats for membrane loading is about 75 ng-atoms/mg protein, compared to 95 ng-atoms/mg protein for the CA rats. This increased capacity for membrane loading seen in the CA rat might result from a greater number of the binding sites responsible for this type of calcium binding.

(b) The Initial Rates of Calcium Uptake

To obtain a more quantitative measure of calcium uptake, the initial rates of this process were obtained by following uptake over the first minute of the time course during which calcium accumulation is linear with respect to time. The results are summarized in Figure 20. The initial rates of calcium uptake both in the presence and absence of phosphate were significantly greater in mitochondria from the CA rats. This increase amounted to 24% (-Pi) and 35% (+Pi).

It is important to establish whether the increases observed in the initial rate of calcium uptake parallel the development of the cold-acclimated state if they are to be implicated in a mechanism of nonshivering thermogenesis. For this reason, the initial rates of calcium uptake were measured on muscle mitochondria from rats exposed to cold and undergoing acclimation

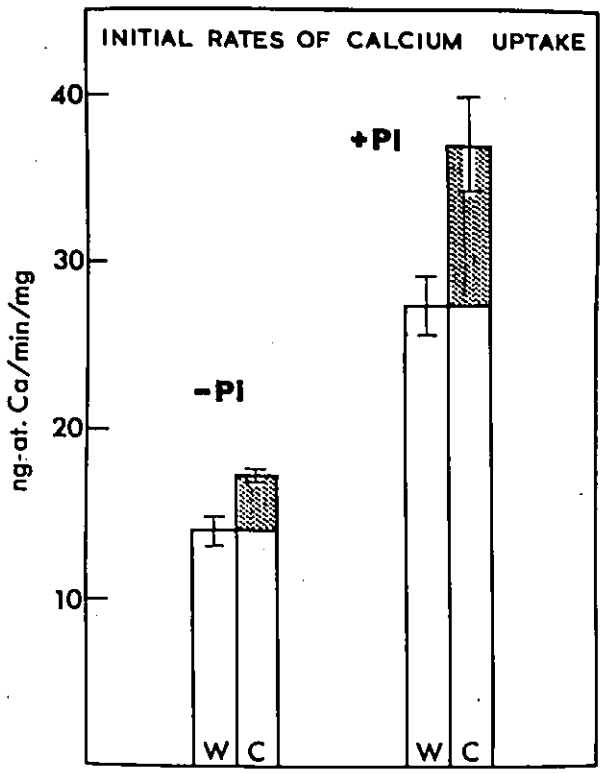

Figure 20. Initial Rates of Calcium Uptake by Muscle Mitochondria from Warm- and Cold-Acclimated Rats

The graph illustrates initial rates of calcium uptake by muscle mitochondria isolated from warm-acclimated (W) and cold-acclimated rats (C) in the presence (+Pi) and absence (-Pi) of 1 mM inorganic phosphate. The shaded portions of the bars indicate significant differences in the initial rate of calcium uptake between mitochondria from warm-acclimated and cold-acclimated rats. Standard errors of the mean are shown as vertical lines on the top of each bar. Values are the means of experiments using 6 individual rats, and were as follows: (a) -Pi: WA; 14.0 ± 0.8; CA, 17.3 ± 0.3 ($P<0.001$); (b) +Pi: WA, 27,4 ± 1.7; CA, 37.0 ± 2.7 ($P<0.02$). Units are ng-atoms Ca^{2+}/min/mg mitochondrial protein. Temperature was 5^{o} C.

to cold. Figure 21 shows the results of this experiment. After one day in the cold, the calcium uptake rate was lower than that seen in the corresponding controls. This is probably the result of the severe cold stress these rats undergo during the first stages of cold exposure. It was also observed that the cold-exposed rats did not eat or drink during their first day in the cold, and this too may have contributed to the decreased calcium uptake rate. Rates of uptake after one, two and three weeks were no different in the cold-exposed rats compared to controls. After five weeks, at which time full acclimation to cold is normally achieved, the initial rate of calcium uptake was greater in the mitochondria from the cold-exposed rats, but the difference was statistically significant ($P<0.05$) only when phosphate was present in the incubation medium. Also shown on the graph for comparative purposes are the results from Figure 20 which represent rats that have lived in the cold or warm for about 100 days.

The most significant finding of this experiment is that the increased rates of calcium uptake are not associated with shivering thermogenesis but only become apparent when the rat is fully cold-acclimated. Ideally, the rate of calcium uptake should increase gradually during cold-acclimation as does the enhanced metabolic response to noradrenaline (see Figure 5, page 17). However, the difference between WA and CA rats is small even after full acclimation to cold, and so to see a gradual increase in rate, one would have to take the average of a large number of experiments over the time course of the acclimation period. This was not done for practical reasons (great amount of time required, large expenditure for rats and chemicals). The experiment reported here, however, does verify that the increased initial rate of calcium uptake seen in the cold-acclimated rat is not present when the

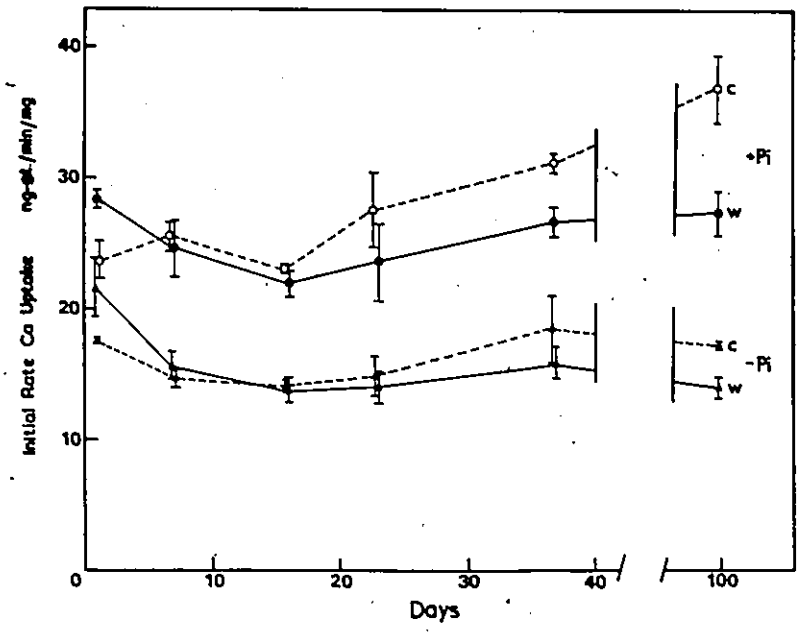

Figure 21. Development of the Increased Initial Rate of Muscle Mitochondrial Calcium Uptake in Rats Undergoing Acclimation to Cold

Rats were placed either in a warm room (27°C) or a cold room (4°C) on day 0. Initial rates of uptake were measured on warm-acclimated rats (W, closed symbols) and cold-exposed rats (C, open symbols) in the presence (circles) and absence (triangles) of 1 mM phosphate on days 1, 7, 16, 23 and 37. Each value represents the mean of experiments using 3 individual rats; standard errors are shown as vertical lines at each point. Also shown, (day 100) are the values obtained in the experiment of Figure 20. Initial rates were determined at 5° C.

animal is using shivering thermogenesis and so may be considered to be truly associated with NST. Further support for this conclusion could be obtained by treating rats with oxytetracycline to prevent the development of the cold-acclimated state and the accompanying alterations in the muscle mitochondria and seeing whether the increased rates of calcium uptake are also abolished.

It is tempting to speculate that one of the adaptive changes that muscle mitochondria undergo during cold-acclimation is a modification of the calcium carrier such that it transports calcium into the mitochondrion at a greater rate, thus contributing to the increased oxygen consumption seen in CA rats. This could come about from an increased number of calcium carriers or an increase in their affinity for calcium. It is significant that the synthesis of at least part of the calcium transport system appears to be controlled by the mitochondrial protein synthesizing apparatus. Carafoli et al. (1973) found that the incorporation of ^{14}C-serine into the Ca^{2+}-binding glycoprotein could be blocked by chloramphenicol, an inhibitor of mitochondrial protein synthesis. The increased turnover of mitochondrial proteins seen in cold-acclimation (Bukowiecki & Himms-Hagen, 1971), and the inhibition by oxytetracycline of the increased number of muscle mitochondria (Himms-Hagen et al., 1975) have pointed out the importance of mitochondrial protein synthesis in cold-acclimation.

Adaptive changes in mitochondrial calcium transport are not without precedent. Bygrave et al. (1975) measured calcium uptake in muscle mitochondria of the developing sheep blowfly Lucilia cuprina. Initial rates were low in mitochondria from flies obtained in the pupal stage, but increased dramatically in newly-emerged flies, being maximal 0-2 hr post-emergence.

Rates then declined as the flies aged and were quite low in the adult blowfly. Interestingly, the respiration rates (state 4 and state 3) of the mitochondria changed in the opposite direction, that is, were low in newly-emerged flies and maximal in adult flies. The reason for the changes in the rates of calcium uptake are not known, but the findings do show that the mitochondrial calcium transport system undergoes adaptive changes associated with the fly's stage of development. It could be a reflection of changing mitochondrial populations because as the fly develops, the mitochondria show a great increase in oxidative capacity in order to provide the large amount of ATP required to support the rapid movement of the flight muscles. In other words, the mitochondria in the mature fly become specialized for ATP synthesis and lose much of their calcium transporting ability.

Another example of a change in mitochondrial calcium transport is the observation of Dorman, Barritt and Bygrave (1975) that elevated blood insulin levels cause a 75% increase in the initial rates of calcium uptake by liver mitochondria and an enhanced ability of these mitochondria to retain accumulated calcium. This change was shown not to be due to hypoglycemia, was cycloheximide-sensitive, and took place 2 hr after insulin administration. The authors proposed that insulin-induced nuclear DNA-directed protein synthesis resulted in altered transport of calcium translocation in mitochondria, possibly as a result of increased production of a protein component of the calcium carrier.

These two examples lend support to the possibility that cold-acclimation could also give rise to increased rates of calcium uptake as a result of an adaptive change in the mitochondrial calcium transport system.

PART E: MEASUREMENT OF THE RELEASE OF CALCIUM BY MUSCLE MITOCHONDRIA FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

In the previous experiment, evidence was presented for an enhanced uptake of calcium by muscle mitochondria from CA rats. If altered uptake of calcium by muscle mitochondria is to be postulated as a thermogenic mechanism in CA rats, it is clear that there must also be altered release of calcium. This set of experiments looks at various aspects of the release of accumulated calcium by mitochondria . First, EGTA is used to compare spontaneous release of calcium by mitochondria from WA and CA rats. Second, the effects of various naturally-occurring releasing agents are tested. These experiments should reveal if muscle mitochondria from CA rats, in addition to displaying a greater rate of uptake of mitochondrial calcium, also release calcium more rapidly, either spontaneously during recycling or under the influence of releasing agents whose concentration is elevated by noradrenaline.

2. Description of the Experiment

Rats used in the experiments reported here had all lived in the warm or cold 2-3 months when sacrificed. The number of rats used and their mean weights for each particular experiment are given in the legends to the figures. The release experiments may be divided into four sections depending on the releasing agent used: (a) EGTA, (b) cyclic AMP, (c) prostaglandin E_1, and (d) Na^+.

3. Results and Discussion

(a) EGTA

The measurement of mitochondrial calcium release in the presence of EGTA is actually a measure of the calcium released during recycling, since

EGTA itself acts only by chelating spontaneously released calcium and does not directly induce release at the level of the calcium transport system (see Literature Review, page 56 , and Reed & Bygrave, 1975b). Figure 22 describes the conditions of the experiment and summarizes the results.

The mitochondria from the CA rat released a greater amount of calcium than mitochondria from the WA rat in the presence of EGTA. Thus both the rates of uptake and release of mitochondrial calcium are enhanced as a result of cold-acclimation. This suggests that muscle mitochondria from CA rats have a greater potential for an increased rate of calcium recycling than those of WA rats. This appears to contradict the findings of the earlier experiment (Part C) in which ruthenium red was used to inhibit calcium recycling. In that experiment, only a small amount of endogenous calcium was present in the mitochondria, while in the experiment reported here mitochondria were first allowed to take up calcium from the medium before release was measured. Both conditions are probably artificial; mitochondria in the intact cell probably contain a quantity of calcium somewhere in between the amounts present in the two experiments. Since calcium release is measured directly in the experiment reported in this section, it is considered to be a more valid indication of calcium recycling than the previous experiment, especially in view of the somewhat anomalous behavior of ruthenium red on mitochondrial respiration rates.

It might also be noted that phosphate was absent from the incubation medium for this experiment. With phosphate present, release of calcium in the presence of EGTA was extremely slow. This was probably due to the removal during uptake of much of the calcium to the mitochondrial matrix as calcium phosphate thus making it less available for recycling. Under

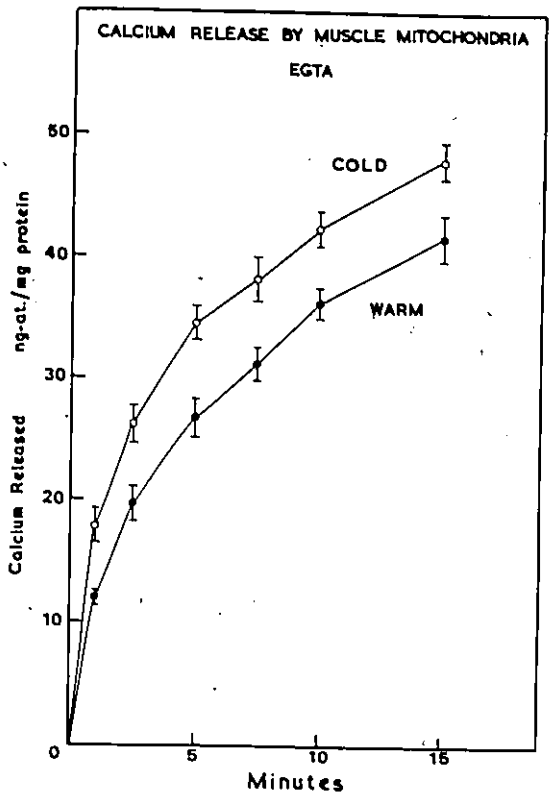

Figure 22. The Effect of EGTA on the Release of Calcium by Muscle Mitochondria from Warm and Cold-Acclimated Rats

Mitochondria were allowed to accumulate ^{45}Ca for 15 min in a medium consisting of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.4, 5 mM pyruvate, 5 mM malate (Tris salts) and 1 mg/ml mitochondrial protein at a temperature of 5°C. A sample was withdrawn to determine the total amount of calcium present in the mitochondria. Then EGTA was added to give a final concentration of 5 mM. Samples were withdrawn at the times indicated on the graph and rapidly filtered. Filters were washed, dried, and counted as described in the Methods to determine the amount of calcium remaining in the mitochondria at each time interval. The amount of calcium released is determined by subtracting this value from the total amount of calcium taken up by the mitochondria before the addition of EGTA. Each point represents the mean ± S.E.M. of experiments using 5 individual warm-acclimated rats weighing 426 ± 38 g (●) and 5 cold-acclimated rats weighing 341 ± 30 g (o). Calcium contents (ng-at./mg protein) at time 0 were: WA: 87.6 ± 4.3 ; CA: 94.5 ± 2.5.

membrane loading conditions the calcium remains in a more mobile pool in association with the mitochondrial inner membrane.

(b) Cyclic AMP

Cyclic AMP in micromolar concentrations was found to cause a rapid release of accumulated calcium from liver, kidney and heart mitochondria (Borle, 1974; Matlib & O'Brien, 1974). However, no success whatsoever was obtained when similar experiments were carried out with muscle mitochondria from both WA and CA rats. Cyclic AMP did not induce calcium release from these mitochondria even though a wide variety of conditions were tried.* Various incubation media were used, including the standard MSH medium used for the uptake and release studies reported in this thesis, as well as the media described by Borle (1974) and Matlib and O'Brien (1974). The presence or absence of phosphate, ATP or protein kinase made no difference. A wide range of cyclic AMP concentrations was tested, but again the mitochondria tenaciously retained their calcium. It must be concluded that either skeletal muscle mitochondria are simply not sensitive to the releasing effects of cyclic AMP, or that the isolation procedure used to prepare these mitochondria destroys their sensitivity to cyclic AMP. The proteolytic digestion step is not at fault because omission of this step still yielded cyclic AMP-insensitive mitochondria. Other workers have also had trouble repeating Borle's findings. For example, Dhalla, Varley and Harrow (1974), working with dog heart mitochondria, reported that "although cyclic AMP has been shown to release calcium from heart mitochondria (Borle, 1974), we have not yet been able to obtain favorable conditions to demonstrate this effect." Also, Carafoli (1976) states in a recent review: "It has recently become clear, however, that the releasing effect of cyclic AMP is not easily repro-

* See Appendix B for data.

ducible and may depend considerably on the experimental conditions employed. In our laboratory, efforts to induce efflux of Ca^{2+} from mitochondria by the cyclic nucleotide have been largely unsuccessful."

It is unfortunate that no cyclic AMP-induced release was demonstrable in muscle mitochondria from CA rats because it would have been a logical way to link the action of noradrenaline on the sarcolemma to an aspect of mitochondrial function, namely an increased respiration rate due to enhanced calcium release followed by reuptake.

(c) Prostaglandin E_1

Prostaglandin E_1 is another naturally occurring substance that causes net calcium release from liver mitochondria (Carafoli & Crovetti, 1973; Malstrom & Carafoli, 1975). The effects of prostaglandins on calcium transport in skeletal or heart muscle mitochondria do not appear to have been studied.

Muscle mitochondria from either WA or CA rats were incubated in 210 mM mannitol, 70 mM sucrose, 5 mM pyruvate, 5 mM malate, 1 mg/ml mitochondrial protein and 200 μM $^{45}CaCl_2$. The medium was buffered either at pH 7.4 with 10 mM HEPES or pH 6.4 with 10 mM imidazole. The temperature used in these experiments was 25°C (Carafoli & Crovetti, 1973).

Mitochondria were allowed to accumulate calcium for 5 min, after which time uptake is complete at 25°C, and then prostaglandin E_1 (PGE_1) was added in a small volume of ethanolic solution to give a final concentration of 5 μM. Alternately, PGE_1 was present in the incubation medium from the beginning before $^{45}CaCl_2$ addition. The calcium contents of the mitochondria at various times were determined as described for previous uptake and release experiments.

At pH 7.4, PGE_1 caused no calcium release, either at a concentration of 5 μM or at other concentrations ranging from 0.1-20 μM.* The presence or absence of phosphate made no difference; neither did altering the temperature or respiratory substrate. Carafoli and Crovetti (1973) found that the PGE_1 effect was optimal under slightly acidic conditions (pH 6.4) and was not apparent at pH's of 7.0-7.4 that are usually used in the study of mitochondrial function.

At pH 6.4, the muscle mitochondria failed to retain their calcium whether PGE_1 was present or not.* The calcium was accumulated normally over the first 2 min but after that it leaked rapidly back into the medium. Thus it was impossible to tell whether PGE_1 had any effect on net calcium release in these experiments. The retention of calcium was not improved by modifying the incubation medium in a variety of ways. This appears to be another way in which muscle mitochondria differ from those of liver, and so unfortunately no conclusions can be drawn about possible differential effects of prostaglandins on muscle mitochondria from WA and CA rats.

(d) Sodium Ion

Figure 23 shows the results of an experiment designed to test the effects of Na^+ on the release of calcium from muscle mitochondria from WA and CA rats. This phenomenon was first reported by Carafoli et al. (1974) for heart mitochondria, and according to these workers does not take place in liver mitochondria to any appreciable extent.

Under certain conditions, Na^+ can induce release of calcium from skeletal muscle mitochondria from both WA and CA rats. Referring to Figure 23, it can be seen that 25 mM Na^+ alone had no effect on net calcium movement. However, if ruthenium red is included in the incubation medium, Na^+ induces a rapid

*See Appendix C for data.

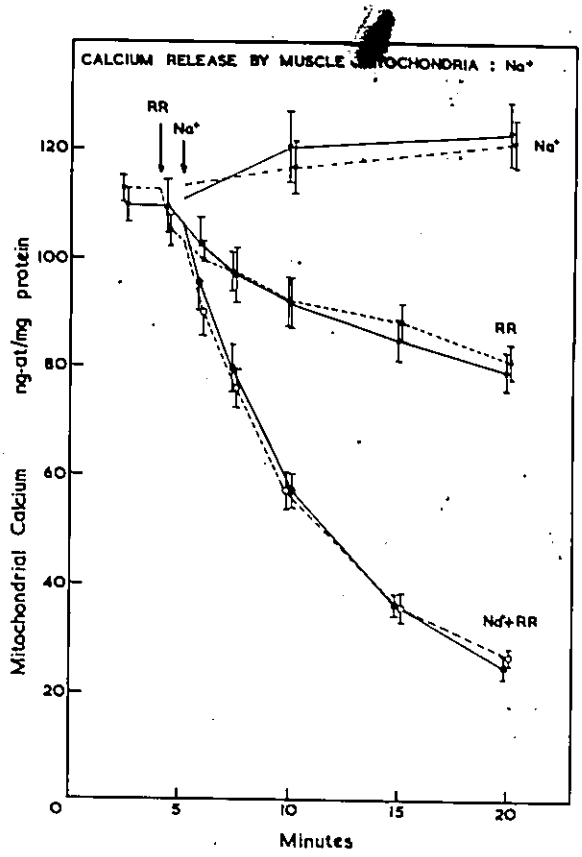

Figure 23. The Effect of Na^+ on the Release of Calcium by Muscle Mitochondria from Warm- and Cold-Acclimated Rats

Mitochondria from warm-acclimated rats (closed symbols, - - - -) and cold-acclimated rats (open symbols, ———) were incubated in 210 mM mannitol, 70 mM sucrose, 10 mM HEPES pH 7.4, 5 mM pyruvate, 5 mM malate at a concentration of 1 mg protein/ml and at a temperature of 25°C. At time 0, 200 μM (final concentration) $^{45}CaCl_2$ was added. Ruthenium red (final concentration 3 μM) was added at 4.5 min, and Na^+ (final concentration 25 mM) was added at 5.0 min. The graph shows the amount of calcium remaining in the mitochondria after adding Na^+ alone (upper curves, ∇), ruthenium red alone (RR, middle curves, Δ), and Na^+ + RR (lower curves, o). Each point represents the mean ± S.E.M. of experiments using 5 individual warm-acclimated rats (380 ± 27 g) and 5 cold-acclimated rats (337 ± 26 g), except for the points on the two upper curves (Na^+, designated as ∇ and ▼) which are the means of only 3 experiments each.

release of mitochondrial calcium, but the rate or extent of this release is no different in mitochondria from CA rats compared to WA rats. In a control experiment, ruthenium red alone induced a certain amount of release, most likely due to the inhibition of calcium reuptake during recycling or perhaps due to an effect of the dye on some other mitochondrial process (see Part C, page 106). Note that at reduced temperatures ($5^{o}C$) ruthenium red did not cause calcium release (see Figure 16). The requirement for ruthenium red or respiratory inhibitors in the incubation medium in order to demonstrate Na^{+}-induced calcium release was also noted by Carafoli et al. (1974).

Release was also seen when the temperature was reduced to $5^{o}C$, when the medium contained 1 mM phosphate, or when the Na^{+} concentration was reduced to 5 mM. However, the rate of release was much lower under these conditions compared to those of Figure 23. Potassium ion caused no calcium release, and when present with ruthenium red induced no more calcium release than ruthenium red alone.

If Na^{+} does induce calcium release and the calcium is immediately taken up again by the mitochondria (upper curves, Figure 23), one would expect a stimulation of mitochondrial respiration by added Na^{+}. It was found that Na^{+} did increase state 4 respiration rates (after Ca^{2+} accumulation) in isolated muscle mitochondria, but other ions such as K^{+} had the same effect. It is likely that this represents an ionic strength effect on the electron transport chain rather than an increase in calcium recycling.

This experiment has successfully demonstrated that Na^{+} can cause calcium release from skeletal muscle mitochondria, and thus it could serve as a physiological releasing agent, enhancing calcium recycling as described in the hypothesis presented in the Literature Review. However, it must be

pointed out that no difference in the response of muscle mitochondria towards Na^+ was observed between WA and CA rats, and so one would have to theorize that noradrenaline causes a greater elevation of Na^+ in the sarcoplasm of a CA rat. Also, ruthenium red must be present to demonstrate an effect of Na^+. This presents a serious problem in relating the observation to the situation in the intact animal.

PART F: MEASUREMENT OF THE HIGH AFFINITY AND LOW AFFINITY CALCIUM BINDING SITES OF MUSCLE MITOCHONDRIA FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

As discussed in the Literature Review (page 53), respiration-inhibited mitochondria possess two classes of calcium binding sites, one of high affinity and the other of low affinity. The high affinity sites are few in number and are thought to represent the mitochondrial calcium carrier. Because the increased rate of calcium uptake observed in muscle mitochondria from CA rats might result from either an increased number of calcium carrier molecules and/or an increase in their affinity for calcium binding, this experiment was undertaken to compare the number and affinity of both the high and low affinity calcium binding sites in muscle mitochondria isolated from WA and CA rats.

2. Description of the Experiment

Six warm-acclimated rats weighing 471 ± 8 g and 6 cold-acclimated rats of weight 390 ± 4 g were used in the binding site experiments. These animals had lived 2-3 months at their respective acclimation temperatures. The techniques used for determining the number and affinity of each class of site is described in detail in the Methods, page 88).

3. Results and Discussion

Figure 24 shows an example of a Scatchard plot derived from measurements on muscle mitochondria obtained from WA rats. It is typically biphasic in nature, indicating that two classes of binding sites of differing affinities are present. The method for calculating the number of each type of site and their affinities for calcium binding are shown in the legend to the figure.

Table V summarizes the binding data obtained from Scatchard plots obtained from experiments using 6 individual WA and 6 CA rats. It is evident that no significant difference exists in either the number or affinity of both classes of binding sites between muscle mitochondria from WA and CA rats.

It is interesting to compare the values obtained for skeletal muscle mitochondria with those obtained for other tissues from various species. Considerable variation exists depending on the source of the mitochondria. Skeletal muscle mitochondria contain more of the high affinity sites than mitochondria from other vertebrate tissues and the dissociation constant is about one order of magnitude higher. Note that a recent study of rat heart mitochondria by Lehninger's group (Jacobus et al., 1975) revealed a rather high value of 80 μM for the dissociation constant of the high affinity sites. This is a much higher figure than previously found in the same laboratory for vertebrate mitochondria. This point will be discussed further below.

Although Lehninger and Carafoli hold the opinion that the high affinity sites represent the calcium carrier, several other workers have challenged this view. Akerman et al. (1974), Southard & Green (1974) and Reed and Bygrave (1974a) all presented evidence that the high affinity sites actually are artifacts of the techniques used to measure them. They argue that inhibition of respiration was incomplete in Lehninger's experiments and that

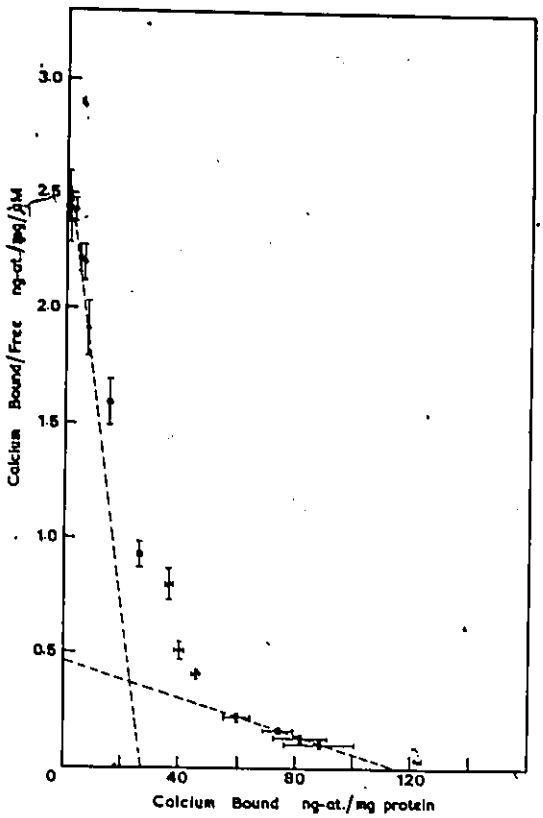

Figure 24. Scatchard Plot of Calcium Binding Data for Muscle Mitochondria Obtained from Warm-Acclimated Rats

The incubation medium consisted of 250 mM sucrose, 2 mM Tris, pH 7.4, 1 mg/ml mitochondrial protein, 0.25 μM rotenone, and 0.225 μM antimycin A. The temperature was $0^{\circ}C$. Each point represents binding data obtained by adding $^{45}CaCl_2$ at various concentrations (1.0-500 ng-atoms/mg protein) and is the mean of experiments using 6 individual warm-acclimated rats. Standard errors of the mean are also shown for the values calcium bound/free (vertical lines) and calcium bound (horizontal lines). The dashed straight lines indicate extrapolations of the high affinity and low affinity portions of the curve. The values obtained were:

(a) high affinity sites: ordinate (nK') = 2.60, abscissa (n) = 26.6
Thus, K' = 0.098 and K = 10.2

(b) low affinity sites: ordinate (nK') = 0.46, abscissa (n) = 114.0
Thus K' = 0.004 and K = 250.

Where n = no. of sites (ng-atoms/mg protein), K' = stability constant (μM^{-1}) and K = dissociation constant (μM).

TABLE V

Comparison of the Number and Affinity of the High Affinity and Low Affinity Binding Sites of Mitochondria from Warm- and Cold Acclimated Rats

	WA	CA	P
High Affinity Sites			
number of sites (ng-atoms/mg protein)	30.5 ± 4.3	38.4 ± 4.8	NS
dissociation constant (μM)	11.4 ± 1.8	13.6 ± 2.3	NS
Low Affinity Sites			
number of sites (ng-atoms/mg protein)	106.8 ± 8.4	103.4 ± 9.7	NS
dissociation constant (μM)	161.0 ± 28.8	136.6 ± 14.9	NS

TABLE VI

Comparison of the Number and Affinity of the High Affinity and Low Affinity Binding Sites of Mitochondria from Different Tissues and Species

Tissue and Species (Reference)	Number of Sites (ng-atoms/mg protein)		Affinity (μM)	
	High	Low	High	Low
Rat liver (Reynafarje & Lehninger, 1969)	1.0	40	0.025	100
Mouse liver (Carafoli & Lehninger, 1971)	4.4	43	1.21	10.4
Rat kidney (Carafoli & Lehninger, 1971)	5	28	1.1	21
Ox adrenal cortex (Carafoli & Lehninger, 1971)	10	60	5.5	100
Yeast (Carafoli & Lehninger, 1971)	none	45	-	13
Blowfly flight muscle (Carafoli & Lehninger, 1971)	none	none	-	-
Sweet potato (Chen & Lehninger, 1973)	7	63	0.58	28
Turnip (Chen & Lehninger, 1973)	39	99	48	200
Rat heart (Jacobus et al., 1975)	8	43	80	1120
Rat skeletal muscle (present study - WA rats).	30.5	106.8	11.4	161

the observed high affinity binding reflects nothing more than residual energy-linked calcium uptake. In the experiments of Jacobus et al. (1975), an attempt was made to overcome this problem by using a higher respiratory inhibitor concentration coupled with a longer preincubation of the mitochondria with the inhibitors than was used in earlier experiments. The result was a lower affinity of the high affinity sites for calcium than was found in earlier work in the same laboratory. This might very well mean that residual energy-lined calcium uptake was inhibited to a greater extent resulting in an increased dissociation constant for the high affinity sites. This finding therefore supports the contention of the critics.

Inhibitors of mitochondrial calcium transport (La^{3+}, ruthenium red) have also been used to estimate the number of calcium carrier molecules (Mela & Chance, 1969 ; Reed & Bygrave, 1974b). Much lower values are obtained by these techniques for the number of carriers than are indicated by measuring the number of high affinity sites.

In summary, the meaning of "high affinity calcium binding" by mitochondria is unclear and so the fact that there was no difference in the number and affinity of these sites between muscle mitochondria from WA and CA rats does not necessarily mean that the number of calcium carrier molecules or affinity of the carrier is not different as a result of cold-acclimation. It would have been better to evaluate the affinity of the calcium transport process by determining the Km for calcium uptake by the usual methods used in enzyme studies, that is by measuring the initial rates of calcium uptake in the presence of increasing calcium concentrations and then performing a double-reciprocal plot (eg. see Reed & Bygrave, 1975a, b).

Low affinity binding by mitochondria is generally considered to represent non-specific calcium binding to mitochondrial membrane components (eg. phos-

pholipids) and so a difference between muscle mitochondria from WA and CA rats would not be expected,

In conclusion, there is no difference in the number or affinity of either high or low affinity calcium binding sites in muscle mitochondria from WA and CA rats. This implies no change in the calcium carrier as a result of cold-acclimation, but the apparent danger of equating high affinity binding with the calcium carrier challenges the validity of this conclusion and leaves open the possibility that differences could be found using other techniques.

CHAPTER V: CONCLUSIONS

The purpose of the research reported in this thesis was to compare various aspects of calcium transport in muscle mitochondria from warm- and cold-acclimated rats in order to find out: (a) whether any differences in this process exist in muscle mitochondria from cold-acclimated rats compared to controls; and (b) if these differences, if found, could provide an explanation for the mechanism of nonshivering thermogenesis in muscle.

The major findings of the study were as follows:

(1) There is no change in the calcium content of isolated muscle mitochondria from cold-acclimated rats.

(2) Small increases in calcium-stimulated respiration rates were observed with certain substrates in muscle mitochondria from cold-acclimated rats.

(3) The recycling of endogenous mitochondrial calcium does not appear to be different in the cold-acclimated rat, based on experiments with ruthenium red and A23187.

(4) Muscle mitochondria from cold-acclimated rats accumulate calcium from the medium at a greater rate than mitochondria from controls. Furthermore, muscle mitochondria develop a greater capacity for membrane loading of calcium as a result of cold-acclimation. The increased initial rate of calcium uptake is seen only in mitochondria from rats fully acclimated to cold and not in rats using largely shivering thermogenesis.

(5) The spontaneous release of accumulated calcium from muscle mitochondria occurs at a higher rate in the cold-acclimated rat when EGTA is present in the medium to prevent reuptake of released calcium.

(6) Of the naturally-occurring calcium releasing agents tested, cyclic AMP

did not induce release of calcium from muscle mitochondria, nor did prostaglandin E_1. Sodium ion induced release if ruthenium red was present in the incubation medium. However, the rate or extent of Na^+-induced calcium release was no different in mitochondria from warm- and cold-acclimated rats. (7) No difference was observed in either the number or affinity of the respiration-inhibited high and low affinity calcium binding sites of muscle mitochondria as a result of cold-acclimation.

The results satisfy the first purpose of the research, that is, cold-acclimation does give rise to changes in the mitochondrial calcium transport system, the most notable change being an increased rate of calcium uptake by the muscle mitochondria. This represents another biochemical difference in these mitochondria, others being increased rates of ADP-stimulated respiration and increased activities of α-glycerophosphate dehydrogenase and adenine nucleotide translocase. The changes found in calcium transport lend further support to the idea that muscle mitochondria undergo "repackaging" during cold-acclimation such that they become more numerous and smaller in size, with this alteration in form being reflected in an alteration in mitochondrial function.

It is not known what changes take place in the mitochondrial calcium transport system as a result of cold-acclimation that allow it to accumulate calcium at a greater rate than that seen in animals not adapted to cold. The most attractive explanation is that a greater number of calcium carriers are synthesized as a result of the adaptive process that takes place during cold-acclimation. It has been demonstrated in this laboratory that cold-acclimation is intimately related to mitochondrial protein synthesis (Himms-Hagen et al., 1975). One could speculate that one of the products of the

increased mitochondrial protein synthesis seen in cold-acclimated rats is a polypeptide component of the calcium transport system. Carafoli et al. (1973) have implicated mitochondrial protein synthesis in the formation of their calcium-binding glycoprotein. It would be interesting to see whether there is a greater rate of turnover of calcium-binding proteins isolated from muscle mitochondria of cold-acclimated rats.

The second purpose of the research, to relate alterations in mitochondrial calcium transport to a biochemical mechanism of nonshivering thermogenesis has only been partially fulfilled and many unanswered questions remain. A hypothesis of nonshivering thermogenesis involving a noradrenaline-induced enhancement of calcium recycling in the muscle mitochondria of cold-acclimated rats was outlined in the Literature Review (see Figure 13, page 74). Noradrenaline was postulated to increase the concentration of a mitochondrial calcium releasing agent in the muscle cell, which would in turn enhance the release of calcium from the mitochondria, making more calcium available for energy-linked uptake. This enhanced recycling of calcium would be an energy-dissipating process and might account in part for the large enhancement of metabolic rate seen in the cold-acclimated rat during noradrenaline infusion. Unfortunately, of the releasing agents tested, only Na^+ induced the release of calcium, and this only occurred in the presence of ruthenium red, which is of course not a naturally-occurring compound. No success was achieved with cyclic AMP and prostaglandin E_1 as releasing agents. Other agents (eg. phosphoenolpyruvate) were not studied. Based on these observations one could now propose that noradrenaline enhances mitochondrial calcium recycling by elevating Na^+ levels in the muscle cell, with Na^+ causing calcium release from the mitochondria. Since no difference was observed in the

rate or extent of calcium release induced by Na^+ in mitochondria from the cold-acclimated rat, noradrenaline would have to elevate Na^+ levels in the sarcoplasm to a greater extent in the cold-acclimated rat than in the warm-acclimated rat. Whether this actually happens is not known, in fact it is not clear what effect noradrenaline has on Na^+ movements in muscle cells.

In the absence of calcium releasing agents, muscle mitochondria from cold-acclimated rats showed an increased rate of calcium uptake and an enhanced release of accumulated calcium in the presence of EGTA. To what extent might these changes contribute to the overall heat produced in non-shivering thermogenesis? The increase in initial rate of calcium uptake was only 35% (in the presence of phosphate), while noradrenaline infusion results in about a four-fold enhancement of metabolic rate in the cold-acclimated rat. It thus seems unlikely that an increase in calcium recycling alone could account for the large increase in metabolic rate characteristic of nonshivering thermogenesis. A method would have to be found for increasing to a much greater extent the availability of calcium for energy-linked uptake by muscle mitochondria in cold-acclimated rats compared to warm-acclimated rats. Conceivably, this could occur by a number of mechanisms; the research reported here has concentrated on ways of increasing calcium availability by enhancing the release of mitochondrial calcium. Possibly, noradrenaline could elevate sarcoplasmic calcium levels by enhancing calcium release from the sarcoplasmic reticulum or by increasing the entry of calcium from the extracellular fluid. Of course, the problem then arises of separating the events of muscle contraction from mitochondrial calcium uptake.

Obviously a considerable amount of research remains to be done before a mechanism of nonshivering thermogenesis in muscle involving calcium recycling can become firmly established. In particular, the effects of catecholamines

on ion movements in intact muscle cells needs to be investigated in both warm- and cold-acclimated rats. Because increasing evidence indicates that the ionic environment plays a major role in regulating metabolic processes, research into that field may provide further clues to the biochemical mechanism of nonshivering thermogenesis.

A problem that should be mentioned with respect to the results reported in this thesis is the possible influence of morphological differences between muscle mitochondria from WA and CA rats. Rates of calcium uptake are expressed in terms of the amount of calcium accumulated per unit mass of mitochondrial protein. However, because mitochondria from the CA rat are smaller than those of the WA rat, there would be more mitochondria (and thus a greater surface area) in the incubation medium in the case of the CA rat even though total protein concentration is the same as for the WA rat. Conceivably, the increased rates of calcium uptake seen in mitochondria from the CA rat may simply be a reflection of an increased area of membrane being made available to the transported ion calcium. Even though a size reduction of the mitochondria would lead to an increased surface area of the outer membrane and the inner boundary membrane, the total surface area of the inner membrane may not necessarily change. The calcium carrier is present in the inner membrane but whether it is localized in one area, such as the inner boundary membrane, has yet to be established. In any event, experiments are currently underway using the Coulter Counter technique to establish the size distribution, volume and surface area of muscle mitochondria from WA and CA rats. Other parameters such as density and water space could also be compared. Such measurements may help to resolve the problem outlined here.

In conclusion, the research reported in this thesis has shown that changes in the muscle mitochondrial calcium transport system take place in the cold-acclimated rat, but incorporating the observed changes into a biochemical mechanism of nonshivering thermogenesis requires further research.

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APPENDIX A

Preparation of Mitochondria for Calcium Determinations by Atomic Absorption Spectrophotometry - Comparison of Acid Extraction and Dry Ashing Techniques

Purpose: to compare two methods for the preparation of mitochondria for the determination of calcium by atomic absorption spectrophotometry:

(a) extraction of calcium from mitochondria by hot HCl.

(b) destruction of total organic matter by dry ashing the mitochondrial protein in a high temperature muffle furnace.

Methods:

(a) HCl Extraction: This method is described on p. 84 of the thesis.

(b) Dry Ashing: Mitochondrial suspensions (about 5 mg protein) were placed in silica crucibles, then evaporated to dryness and charred under an infra-red lamp. The samples were then ashed overnight in a muffle furnace at 450° C. The ash was dissolved in 2.0 ml of 0.5 N HCl containing 1% $LaCl_3$ and the calcium concentration determined by atomic absorption spectrophotometry.

Results: The table gives calcium contents (ng-atoms/mg protein) of mitochondria from WA and CA rats:

		WA	CA
(a)	Acid extraction (n=8)	14.5 ± 1.4	15.1 ± 1.2
(b)	Dry ashing (n=3)	14.8 ± 5.9	16.3 ± 3.8

Conclusions: It is clear that calcium concentrations determined in the dry ashed mitochondrial preparations are not different from those determined following acid extraction. Because it is simpler to perform and gives more reproducible results, the acid extraction method was chosen for the investigations reported in this thesis.

APPENDIX B

Lack of Effect of Cyclic AMP on the Release of Calcium by Muscle Mitochondria and on Mitochondrial Respiration Rates

(a) Calcium Release

Release in the presence of cyclic AMP was measured using the principles described in the Materials and Methods (see pp. 87-88). The results of a representative experiment are presented here:

Mitochondria (1 mg/ml) were allowed to accumulate calcium for 15 min in an incubation medium consisting of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM pyruvate, 5 mM malate, 200 μM $^{45}CaCl_2$ and 1 mM phosphate. Total volume was 2 ml and the temperature was 5° C. After completion of calcium uptake, one of the following was added:

(i) no additions (control)

(ii) cyclic AMP (3 μM final concentration - Borle (1974))

(iii) ruthenium red (3 μM final concentration)

(iv) cyclic AMP + ruthenium red

The following table gives the calcium content of the mitochondria (ng-atoms/mg protein) measured at various times after addition of cyclic AMP and/or ruthenium red.

TIME (min)	CONTROL		cAMP		RR		cAMP + RR	
	WA	CA	WA	CA	WA	CA	WA	CA
0	190.6	185.4	198.1	194.7	193.5	189.5	195.1	199.3
1.0	192.0	180.6	194.7	194.6	192.6	192.8	188.4	192.9
2.5	195.5	182.7	194.9	194.5	182.7	189.2	183.9	190.9
5.0	194.5	192.7	193.0	194.0	178.5	185.4	178.8	185.2
10.0	194.4	185.0	191.1	191.7	177.4	177.2	168.8	173.7

The results show that cyclic AMP does not cause release of calcium from the muscle mitochondria from either WA or CA rats. The addition of ruthenium red along with cyclic AMP caused no more release than did ruthenium red alone, thus arguing against the possibility that cyclic AMP is causing release of calcium followed by a rapid reuptake.

A similar lack of effect of cyclic AMP was noted in experiments utilizing a variety of other conditions, for example:

- different cyclic AMP concentrations (0.01, 0.1, 1.0, 2.0, 3.0, 5.0, 10.0, 100 μM).
- presence of protein kinase (0.1 mg/ml)
- presence of ATP (0.5 mM)
- different initial calcium and phosphate concentrations, including no phosphate.
- different incubation temperatures (5, 25, 37° C.).

(b) Respiration Rate

Cyclic AMP (3 μM final concentration) was added to muscle mitochondria respiring in state 4 either before calcium addition or after calcium accumulation. The incubation medium was 80 mM KCl, 10 mM Tris (pH 7.4), 0.5 mM phosphate, 0.2% bovine serum albumin, 5 mM pyruvate, 5 mM malate, mitochondrial protein 1 mg/ml. Total volume of the medium was 2.0 ml and the temperature was 37° C. Calcium was added to give a final concentration of 0.2 mM.

	Respiration Rate (ng-atoms O/min/mg)	
	WA	CA
(i) State 4 (before Ca^{2+})	43.1	49.5
Rate in presence of cAMP	43.1	49.5
(ii) State 4 (after Ca^{2+} accumulation)	44.6	50.1
Rate in presence of c AMP	44.6	50.1

Cyclic AMP thus does not alter the rate of respiration of muscle mitochondria. This also argues against a cyclic AMP-induced release of calcium followed by rapid reuptake, since this process would be expected to stimulate respiration.

APPENDIX C

The Effects of Prostaglandin E_1 on Calcium Retention by Muscle Mitochondria

Release in the presence of prostaglandin E_1 was measured according to the techniques described in the Materials and Methods (pp. 87-88).

Mitochondria (1 mg/ml) were loaded with calcium in a medium consisting of 210 mM mannitol, 70 mM sucrose, 10 mM HEPES (pH 7.4), 5 mM pyruvate, 5 mM malate, 200 μM $^{45}CaCl_2$, and in the presence or absence of 1 mM phosphate. The total volume of the incubation medium was 2.0 ml and the temperature was 5° C. Prostaglandin E_1 was then added at a final concentration of 5 μM, and the samples were withdrawn at timed intervals thereafter and the calcium concentration of the mitochondria determined. The following table summarizes the results; calcium concentrations are expressed as ng.-atoms/mg protein. Note that the ruthenium red (RR) concentration was 3 μM.

TIME (min.)	Phosphate Present				Phosphate Absent			
	-RR		+RR		-RR		+RR	
	WA	CA	WA	CA	WA	CA	WA	CA
0	187.9	194.6	193.5	197.0	86.3	87.5	81.8	87.6
1.0	184.1	194.3	196.7	192.2	85.2	87.6	82.2	83.9
2.5	189.9	192.7	188.2	186.7	87.9	89.6	84.3	85.8
5.0	185.3	195.3	184.4	181.1	85.8	93.1	81.6	85.6
10.0	185.0	192.0	172.6	166.4	90.1	92.8	82.8	78.7
15.0	181.8	182.2	161.5	157.8	88.5	88.6	77.7	85.1

Under these conditions no PGE-induced release was seen. The release evident when phosphate and ruthenium red are included in the incubation medium is no more than that normally seen under the same conditions in the absence of

PGE. A similar lack of effect of PGE was found under the following conditions:

(i) Temperature of 25° C.

(ii) PGE concentrations of 1, 10 and 20 μM.

(iii) PGE added to the incubation medium before calcium.

(iv) Reduction of the pH to 6.4.

Carafoli and Crovetti (1973) reported that the effect of PGE on calcium release was more evident at pH 6.4 than at 7.4. As noted above, reduction of the pH to 6.4 was without effect using the described incubation medium. However, Carafoli and Crovetti used an ionic medium rather than a sucrose medium. The experiment was therefore repeated under the following conditions: 100 mM KCl, 10 mM succinate, 10 mM imidazole (pH 6.4), 1 mg/ml mitochondrial protein, 5 μM PGE in a total volume of 2.0 ml and at a temperature of 25° C. Mitochondria were incubated for 5 min in the presence of 200 μM $^{45}CaCl_2$, and the PGE was added at time = 0. The results are summarized in the following table; calcium contents are expressed as ng-atoms/mg protein.

Time (min.) after PGE addition	Control Uptake (no PGE) WA	CA	PGE Added WA	CA
1.0	35.7	35.7	16.4	29.4
2.5	36.2	35.4	15.4	27.3
5.0	27.9	26.3	9.2	22.3
7.5	26.5	22.4	10.1	19.5
10.0	24.5	21.6	9.5	18.4
15.0	22.1	13.7	7.0	13.6
20.0	14.1	10.4	6.4	9.5

Under these conditions the mitochondria fail to retain their calcium and so the effect of PGE cannot be evaluated.