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IONIC CHANGES IN BROWN ADIPOSE TISSUE MITOCHONDRIA DURING ACCLIMATION TO COLD IN RATS

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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 Master of Science.

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SUMMARY

A rat which is exposed to cold is able to produce heat and maintain its body temperature by shivering. However, if the rat is allowed to remain in the cold for 3 or 4 weeks, the rat undergoes an adaptive change allowing it to produce heat and maintain its body temperature without shivering. An animal capable of producing heat without shivering is said to be cold-acclimated and the term nonshivering thermogenesis has been used to describe the process which allows for the increase in heat production.

The mechanism of heat production by shivering is fairly well understood and appears to be similar to heat production during light excercise. In contrast to this, the mechanism of nonshivering thermogenesis is not well understood. It is known that noradrenaline, secreted by an animal using nonshivering thermogenesis, is responsible for switching on the process and that the response takes place largely in the brown adipose tissue. The capacity of an animal, such as the rat, to respond to noradrenaline by an increase in heat production increases during acclimation to cold. Cold-acclimated rats have a large capacity for nonshivering thermogenesis.

In brown adipose tissue, as in other tissues, heat production is the result of combustion of substrate molecules during increased respiration. For this reason, the mitochondrion of brown adipose tissue has been the logical place in which

to look for biochemical differences that might explain an increased capacity for nonshivering thermogenesis. Brown adipose tissue mitochondria isolated from cold-acclimated rats have been found to be different morphologically and biochemically when compared with brown adipose tissue mitochondria isolated from warm-acclimated rats; however, the differences observed cannot explain the mechanism of nonshivering thermogenesis in this tissue.

Most of the recent evidence indicates that heat production by nonshivering thermogenesis involves a loose-coupling type of mechanism. This means that the rate of electron transport is no longer dependent on the availability of ADP but is controlled by other factors. A loose-coupling type of mechanism can occur when the cycling of ions is promoted. Since it has been shown that removal of magnesium ions from mitochondria causes an increase in the permeability of the mitochondrial inner membrane to ions and stimulates ion cycling it was of interest to investigate the possible role of magnesium ions in nonshivering thermogenesis.

The purpose of the work described in this thesis was to investigate the role, if any, of mitochondrial magnesium ions in nonshivering thermogenesis. In order to do this, brown adipose tissue mitochondria isolated from warm-acclimated rats were compared with mitochondria isolated from cold-acclimated rats to find out whether any differences existed and whether these differences, if found, might give a clue to the mechanism of nonshivering thermogenesis in brown adipose tissue

of the cold-acclimated rat. Experiments were also performed using mitochondria isolated from a tissue, the liver, known not to undergo changes in thermogenic activity during cold-acclimation. This was done in order to determine whether any changes in interscapular brown adipose tissue mitochondria following cold-acclimation were indeed related to the altered thermogenic activity of this tissue observed in the cold-acclimated rat.

The major findings of the study were as follows:

1) Interscapular brown adipose tissue (IBAT) mitochondria isolated from cold-acclimated rats display a greater rate of calcium-induced swelling than IBAT mitochondria isolated from warm-acclimated controls.

2) There is a decrease in the level of endogenous magnesium in IBAT mitochondria of cold-acclimated rats.

3) Only 5% of the total content of magnesium is located in the intermembrane space of IBAT mitochondria isolated from warm- and cold-acclimated rats.

4) Liver mitochondria isolated from warm- and cold-acclimated rats show no differences in calcium-induced swelling and have similar levels of endogenous magnesium.

The results suggest that one of the adaptive changes that brown adipose tissue mitochondria undergo during cold-acclimation is loss of endogenous magnesium. This loss is not observed in liver mitochondria isolated from cold-acclimated rats. Although the results suggest that the observed loss of brown adipose tissue mitochondrial magnesium in the cold-

acclimated rat may have a role in the control of the mechanism of nonshivering thermogenesis in IBAT mitochondria, further work is necessary to establish the nature of this role.

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ABREVIATIONS

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BAT	brown adipose tissue
CA	cold-acclimated
CNS	central nervous system
DNP	2,4-dinitrophenol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(aminoethylether) N,N'-tetraacetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IBAT	interscapular brown adipose tissue
NA	noradrenaline
NEM	N-ethylmaleimide
NST	nonshivering thermogenesis
SNS	sympathetic nervous system
WA	warm-acclimated

CHAPTER I: . INTRODUCTION AND STATEMENT OF THE PROBLEM

An animal which is placed in a cold environment must maintain its body temperature in order to survive. The first defence mechanism of a rat against the cold is heat production through shivering. However, 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 using this form of heat production is said to be cold-acclimated.

In contrast to earlier conclusions, brown adipose tissue appears to be the major site of nonshivering thermogenesis. The contribution of skeletal muscle to the overall heat produced in nonshivering thermogenesis appears to be small. Brown adipose tissue is an organ which proliferates during acclimation to cold and is especially adapted for producing heat.

An animal using nonshivering thermogenesis (i.e. a cold-acclimated rat) responds calorigenically to infused noradrenaline to a much greater extent than an animal not adapted to use nonshivering thermogenesis (i.e. a warm-acclimated rat). Nonshivering thermogenesis is known to be switched on by noradrenaline secreted by sympathetic nerves in the animal exposed to cold. The large enhancement of the metabolic rate in response to catecholamines is an important characteristic of animals possessing the adaptation for nonshivering thermo-

genesis.

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. In brown adipose tissue, as in other tissues, heat production is the result of combustion of substrate molecules, i.e. of respiration. An increase in respiration results in an increase in heat production. For this reason, the mitochondrion is the obvious place in which to look for biochemical differences that might explain an increased capacity for nonshivering thermogenesis. With this in mind studies have been undertaken to study brown adipose tissue mitochondria from cold-acclimated rats since brown adipose tissue is the major site of heat production in nonshivering thermogenesis in these animals in which the capacity for this form of heat production is increased.

Brown adipose tissue mitochondria undergo alterations associated with the development of nonshivering thermogenesis in the cold-acclimated rat. The mass of the tissue itself grows in response to cold. The mitochondria become larger and more numerous per cell. They have more tightly packed cristae and have a greater respiratory capacity than mitochondria obtained from other tissues. When mitochondria are isolated from brown adipose tissue they are usually uncoupled or loosely coupled. Mitochondrial binding of ADP or GDP, which has been shown to be necessary to recouple these mitochondria, is higher in mitochondria isolated from cold-acclimated rats.

The binding site of these purine nucleotides is known to be a polypeptide of molecular weight 32,000. It has been observed that the amount of this mitochondrial protein also increases during cold-acclimation. Brown adipose tissue 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.

Ion transport is known to have the potential to provide a significant thermogenic process. The uptake of many ions is an energy-requiring process. If the accumulated ion is allowed to be released it would then be available for active re-uptake. The operation of such a futile cycle could represent a major thermogenic process. One method by which an accumulated ion could be released would be if the permeability of the membrane were to increase. The removal of mitochondrial magnesium has been shown to increase the permeability of mitochondrial membranes and hence could be implicated in a given futile cycle.

The purpose of the research reported in this thesis is to compare one aspect of ion transport, namely calcium-induced swelling, and to measure the endogenous magnesium content in brown adipose tissue mitochondria isolated from cold- and warm-acclimated rats 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 brown adipose tissue of the cold-acclimated rat. Similar

experiments were performed using mitochondria isolated from a tissue known not to undergo changes in thermogenic activity during cold-acclimation, namely, the liver. This was done to ensure that any changes in brown adipose tissue mitochondria isolated from warm and cold-acclimated rats were indeed related to the high thermogenic activity of this tissue observed in the cold-acclimated rat. The problems examined are as follows:

(a) Comparison of calcium-induced swelling in mitochondria from warm- and cold-acclimated rats isolated from the tissue known to be involved in nonshivering thermogenesis, namely, brown adipose tissue.

(b) Comparison of the magnesium content of brown adipose tissue mitochondria isolated from warm- and cold-acclimated rats.

(c) Comparison of calcium-induced swelling in mitochondria from warm- and cold-acclimated rats isolated from a tissue known not to be involved in nonshivering thermogenesis, namely, the liver.

(d) Comparison of the magnesium content of liver mitochondria isolated from warm- and cold-acclimated rats.

The literature review that follows is divided into three parts. In part A, the characteristics of cold-acclimation and nonshivering thermogenesis are discussed and the role of the sympathetic nervous system in the process and the biochemical mechanism of heat production in brown adipose

tissue are reviewed. Part B discusses how ion transport can provide a potentially significant thermogenic mechanism and how such a mechanism might be regulated. The role of ions in acclimation to cold and the current knowledge of the ionic changes which occur in mitochondria as a result of cold-acclimation are discussed in Part C. 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

An animal which is exposed to the cold must maintain its body temperature in order to survive. This maintenance of body temperature is usually achieved by decreasing heat loss, through piloerection and peripheral vasoconstriction, and by increasing heat production. This review will concern itself only with the mechanism of increasing heat production.

There are two distinct mechanisms which enable an animal to increase heat production when exposed to the cold, shivering thermogenesis and nonshivering thermogenesis. As the name implies, shivering thermogenesis is the production of heat in muscle by metabolic processes that are accelerated by shivering. The mechanism of heat production through shivering thermogenesis is fairly well understood and appears to be similar to heat production during light excercise. In contrast to this, the mechanism of nonshivering thermogenesis is not well understood. Although much is known about it, the biochemical processes which allow for its large production of heat are still not clear. Before discussing the current concepts concerning nonshivering thermogenesis it is necessary to review briefly the role of shivering thermogenesis in heat production.

I. Cold Stress and Shivering Thermogenesis

When a rat is placed in the cold (4°C) it responds by shivering and its body temperature is maintained by this

process. Shivering has been defined by Hemingway (1963) as "a rhythmic involuntary contraction of voluntary muscle which is activated as a response to cold and is under the control of the central nervous system". The mechansim of shivering thermogenesis is relatively well understood. The ATPase associated with shivering produces an increased supply of ADP to the mitochondrion. ADP then stimulates the oxygen consumption of the tightly coupled muscle mitochondria and its own rephosphorylation into ATP (Owen & Wilson, 1974). Increased quantities of heat are produced during the breakdown of ATP and during the stimulated respiration of the mitochondrion.

The first and essential stimulus for shivering thermogenesis is the release of acetylcholine by motor nerves. Cottle and Carlson (1956) showed that if a warm-acclimated rat is treated with tubocurarine, a drug which paralyzes skeletal muscle by blocking synaptic transmission at the neuromuscular end plate, and then exposed to cold, the animal quickly becomes hypothermic and dies. The cold stress also activates the sympathetic nervous system so that the sympathetic nerve endings and the adrenal medulla secrete noradrenaline and adrenaline respectively. The released catecholamines, in turn, perform two important functions. They give rise to piloerection which reduces heat loss and secondly, they mobilize 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 formed by gluconeogenesis is also used (Himms-Hagen, 1972).

2. Cold-Acclimation and Nonshivering Thermogenesis

Thus the first defence mechanism of a rat against the cold is heat production through shivering. However, if the rat is allowed to remain in the cold, shivering gradually decreases and disappears by the 3rd or 4th week. The rat is then said to be cold-acclimated. The decrease in shivering with time is shown in Fig. 1 (Hart et al., 1956).

Since the rat is still in the cold another mechanism of heat production must be present. This alternative method has been called "nonshivering thermogenesis" and its biochemical mechanisms have not as yet been completely elucidated. The fact that shivering thermogenesis and nonshivering thermogenesis are separate entities is shown by the experiments of Cottle and Carlson (1956) who administered tubocurarine to a cold-acclimated rat and noted that the animal was still able to maintain a high metabolic rate and a normal body temperature in the cold.

Jansky (1973) defined nonshivering thermogenesis as a "heat-production mechanism liberating chemical energy due to processes which do not involve muscular contractions". He further stated that most of the heat production under conditions of basal metabolism, i.e. at rest and at the temperature of the thermoneutral zone, is due to obligatory or basal thermogenesis. However, the additional heat production which occurs at ambient temperatures below the thermoneutral zone is

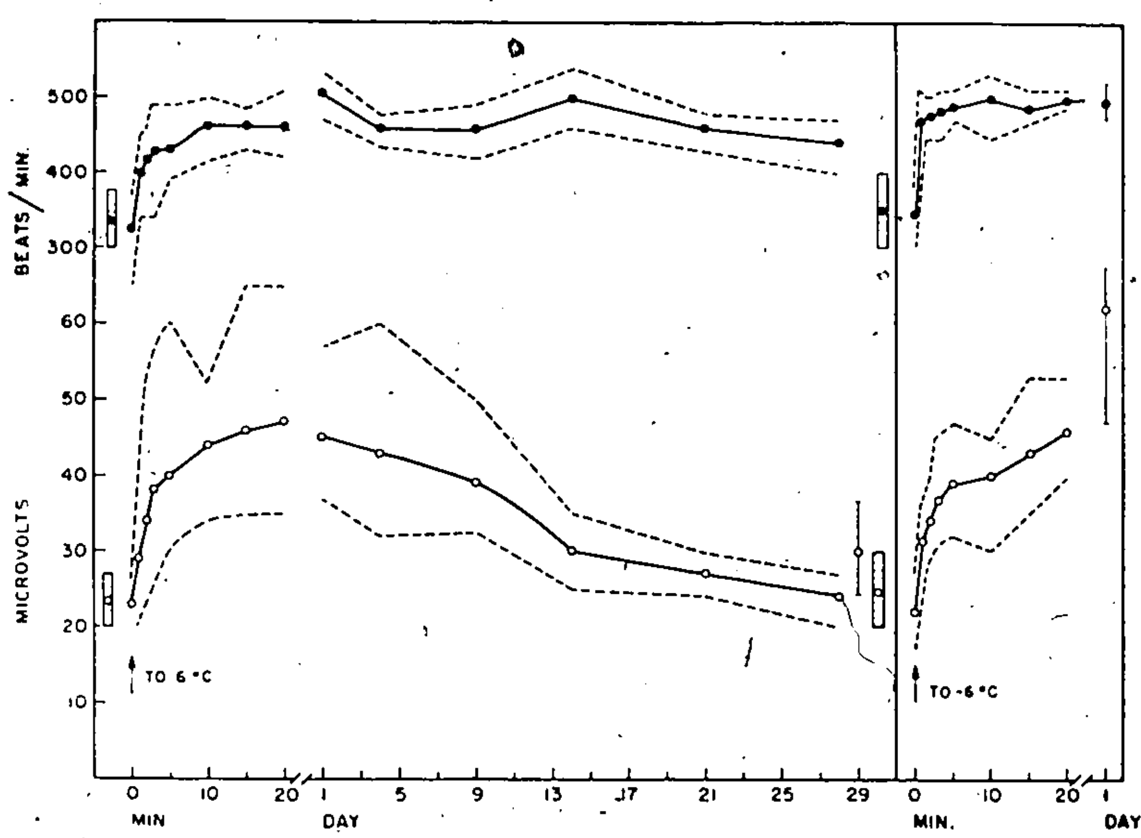

Fig. 1. Increase and Decrease in Shivering as Measured by Muscle Electrical Activity, During Acclimation to Cold.

Muscle electrical activity as related to time of exposure to 6°C is shown (solid line) in the left-hand part of the diagram (lower tracing). Broken lines show range of variation. Vertical bars at 0 and 29 days show muscle electrical activity in warm-acclimated rats living at 30°C. Upper tracing shows heart rate. At 30 days cold-acclimated rats were moved to -6°C (right-hand part of the diagram). (From Hart et al., 1956).

due to regulatory thermogenesis. This review will only be concerned with regulatory thermogenesis (nonshivering thermogenesis, NST).

NST has two important characteristics. The first is that it is an adaptive process. This is indicated by the observation that it takes about 3 or 4 weeks of cold exposure before the rat can rely on NST as a source of heat. Other species such as the rabbit and guinea pig must also remain in the cold for a relatively long time to be able to produce heat by NST. It is also used by hibernators (e.g. bat, hamster, ground squirrel) particularly during arousal from hibernation. The newborn of most species (e.g. rabbit, rat, guinea pig, human) also use NST as a heat producing mechanism, but this ability is lost quite soon after birth and is absent in the adult animal (see review by Himms-Hagen, 1970).

The second important characteristic of NST is that it is a facultative process. This means that it can be switched on and off according to the needs of the animal (Depocas, 1960 a). If a cold-acclimated rat is transferred from a cold environment to a warm environment it will turn off NST; if the same animal is then transferred back to the cold it has the ability to turn on and utilize NST once more.

The physiological significance of NST is most evident in newborn animals. Some animals, such as the rat, are born immature and are incapable of shivering and so they must rely entirely on NST to prevent hypothermia. Another example is the

harp seal pups born on open ice floes in the arctic seas. These newborn animals are exposed to a thermogradient approaching 70°C and NST plays a decisive role in their defence against this extreme cold stress (Grav et al., 1974).

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

In contrast to the regulation of shivering thermogenesis by the central nervous system, NST appears to be regulated by the sympathetic nervous system. It was mentioned earlier that this system was important in shivering thermogenesis by reducing heat loss and by mobilizing the necessary substrates. While this is still true for an animal which is using NST, there is ample evidence which indicates that noradrenaline, secreted from sympathetic nerve endings, plays a direct role in both the adaptive and facultative properties which are characteristic of NST (Himms-Hagen, 1975).

Two lines of evidence for the importance of the sympathetic nervous system will be discussed.

(a) The Mimicking Effects of Catecholamines

Hsieh and Carlson (1957) were the first to suggest that noradrenaline was the mediator in the regulation of NST by noting the effects of infused noradrenaline on oxygen consumption. Fig. 2 (Depocas et al., 1978) illustrates the dramatic difference in oxygen consumption rates between warm-acclimated and cold-acclimated rats during such an infusion. The increase in oxygen consumption in response to noradrenaline of the cold-

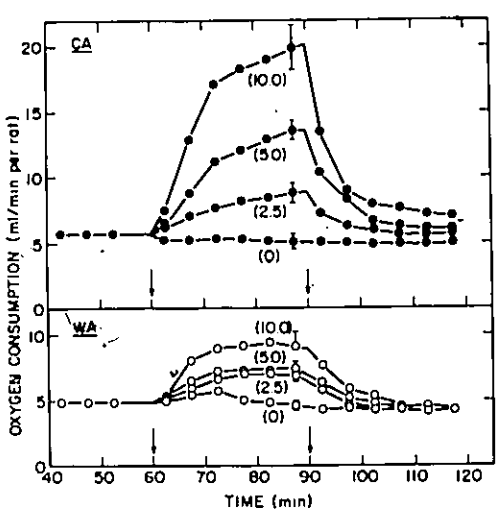

Fig. 2. The Rates of Oxygen Consumption Before, During, and After Infusion of Noradrenaline into Barbital-Sedated Warm- and Cold-Acclimated Rats.

Average oxygen consumption (averaged for each animal during 5-min intervals from readings made at 30-s intervals) measured in barbital-sedated WA (O) or CA (●) rats before, during and after continuous infusion of NA bitartrate at rates of 0, 2.5, 5.0 and 10.0 ng (free base) min^{-1} $g^{0.74}$ body weight. Infusions into the tail vein were started at 60 min and stopped at 90 min. There were three rats in each group. The standard error of the mean is plotted for each group at 87.5 min.

acclimated rats illustrates the most characteristic property of an animal using NST as a mechanism of heat production. The facultative nature of NST is illustrated by the rapid increase in oxygen uptake when the infusion starts and the rapid decrease when the infusion stops.

The possibility that the enhancement of the calorigenic response is due to an increased sensitivity to noradrenaline is eliminated by the fact that the dose-response curve is the same between warm- and cold-acclimated rats (Himms-Hagen et al., 1972). Moreover, measurements of plasma noradrenaline levels during infusion of noradrenaline into warm- and cold-acclimated rats at room temperature (Depocas et al., 1978) have shown that at a given infusion rate, the arterial concentration of noradrenaline is lower in cold-acclimated rats than in warm-acclimated rats (Table 1). This finding disagrees with the suggestion put forward by Leblanc and Pouliot (1964) that higher circulating levels of NA could be responsible for the increased calorigenic response to NA in the cold-acclimated rats. Table 1 indicates the molar steady-state concentrations of plasma NA during its infusion into warm- and cold-acclimated rats. The values reported in Table 1 are estimates of the concentrations of NA acting on the receptors for the various degree of activation shown in Fig. 2. In rats receiving no NA, the plasma levels were 2.0 and 1.4 nM in cold- and warm-acclimated rats respectively. In rats receiving 10 ng NA $min^{-1} g^{-0.74}$ body weight the corresponding

TABLE I

Average Molar Concentrations of NA in Arterial Plasma During Intravenous Infusion of NA into Barbital-Sedated Warm- and Cold-Acclimated Rats.

Infusion rate	Concentrations, nM	
ng $min^{-1} g^{-0.74}$	WA	CA
0	1.38 ± 0.91	1.93 ± 0.58
2.5	28.5 ± 1.3	19.3 ± 1.2
5.0	48.1 ± 0.2	33.2 ± 3.3
10.0	108.0 ± 4.0	59.7 ± 4.3

(From Depocas et al., 1978)

values were 60 nM and 110 nM. These values are of the same order of magnitude as those found to be effective in increasing the respiratory rate of isolated hamster brown adipose tissue cells (Petterson & Vallin, 1976) and of perifused interscapular brown adipose tissue fragments (Seydoux & Girardier, 1978).

Depocas (1960 b) provided further evidence that NA was involved in the adaptation of the rat to the cold by noting that the time course of development of increased response to NA (Figure 3) paralleled other changes, related to heat production, which have been observed during development of cold-acclimation, such as an increase in food consumption, increase in survival at lethal cold, increase in resistance to body cooling and replacement of shivering by nonshivering means of heat production (Hart, 1958). That 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 is evident when one compares the time course of the increased response to NA (Figure 3) to the time course of the decrease in muscle electrical activity as a function of time of exposure to 6°C (Figure 1). Muscle electrical activity was found to return to the basal level observed in rats at 30°C in about 4 weeks. This corresponds approximately to the time required for development of maximal capacity to respond to NA.

Bartunkova et al. (1971) noted that if a cold-acclimated

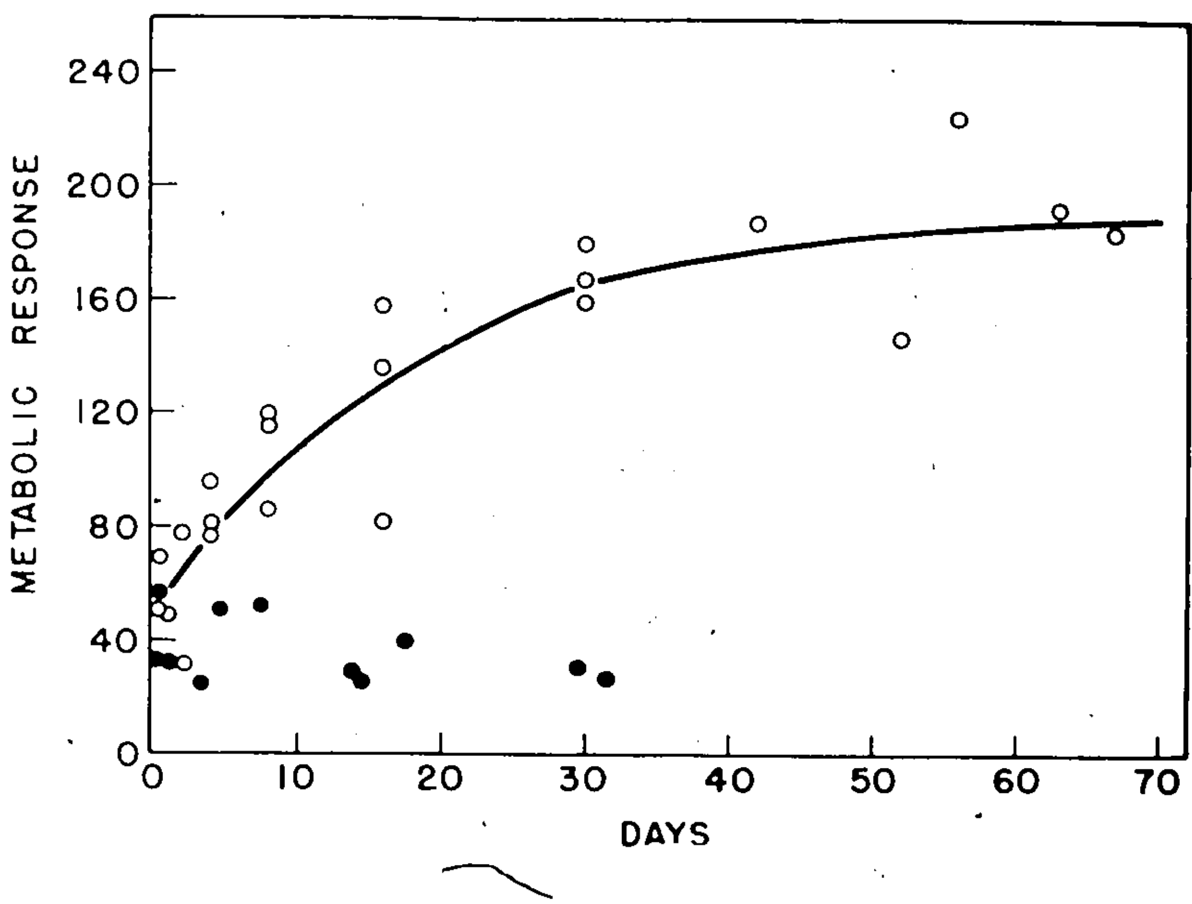

Figure 3. Development of Enhanced Calorigenic Response to Noradrenaline During Acclimation to Cold

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 b)

rat is removed from the cold and placed in a warm environment there was a gradual disappearance of the enhanced metabolic response to NA which paralleled the loss of ability to produce heat by NST.

(b) Activation of the Sympathetic Nervous System

It is evident that if NA is directly involved in NST then the secretory activity of peripheral sympathetic nerves during exposure of mammals to cold should be increased. If this is so then part of the secreted NA which escapes reuptake or metabolic degradation should overflow into the body fluids.

The major difficulty, until recently, in measuring plasma levels of NA had been the low amounts which were present. Earlier studies which attempted to demonstrate that the sympathetic nervous system was activated during cold exposure had been based on chronic procedures such as measurements of the NA levels in urine which had to be collected over many hours or days (Leduc, 1961; Shum et al., 1969). Using the recently developed radioenzymatic procedure, Depocas and Behrens (1978) measured the plasma NA levels of warm-acclimated and cold-acclimated rats at three different temperatures. As is shown in Fig. 4 there was a 3-fold increase in NA concentrations in the plasma on exposure to 5.5°C and an approximately 9-fold increase at -18.2°C which took place in both warm-acclimated and cold-acclimated rats. Another experiment performed under the same exposure conditions, but with more frequent sampling of blood, indicated that the changes

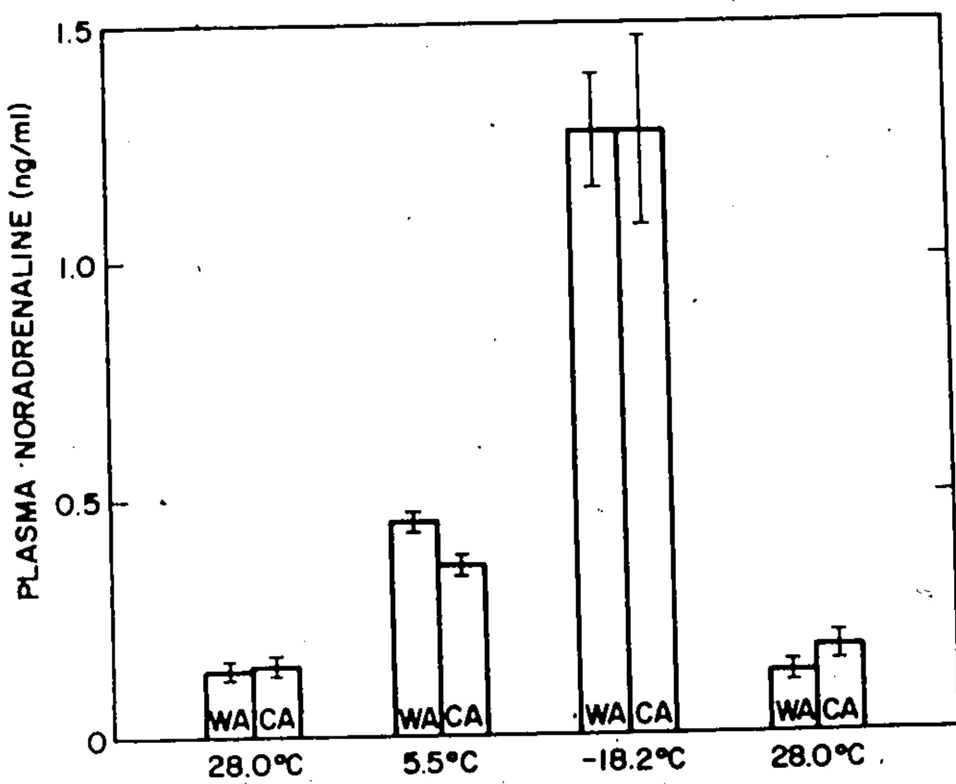

Figure 4. Average Plasma NA Concentrations in Warm- and Cold-Acclimated Rats as a Function of Temperature

The figure compares the average concentration of NA in plasma of 4 WA and 4 CA rats exposed to various degrees of cold. Rats were initially at 28°C and then exposed to 5.5°C and -18.2°C for intervals of 30 min and then returned to 28°C.

(From Depocas & Behrens, 1978)

observed in Fig, 4 occurred during the first few minutes of exposure to cold and that these increases were maintained as long as the exposure was maintained. In other words when the rats were transferred from 28°C to 4°C the 3 fold increase in the NA level in their plasma occurred during the first few minutes of cold exposure. If the rats were then taken from 4°C and placed at -18.2°C a further 3 fold increase occurred a few minutes later.

There results, therefore provide strong evidence which indicates that NA secreted from sympathetic nerves plays a direct role in NST.

4. <u>The Site of Nonshivering Thermogenesis</u>

There has been a long controversy about the site of heat production during NST and until recently, much of the evidence pointed to skeletal muscle rather than brown adipose tissue (see section 5) as the major site of NST. Different approaches were taken in order to evaluate the increase in rate of O_2 consumption of individual organs and to compare it with the increase in the rate of O_2 consumption of that specifically produced by NST in the cold-acclimated rat.

The different approaches used were:

(a) removal of the tissue, either surgically or functionally to assess its contribution to the total O_2 consumption.

(b) measurement of cytochrome oxidase activity to assess the metabolic capacity of a given tissue.

(c) blood flow measurements together with arteriovenous

differences in blood oxygen.

The first approach has been useful in assessing the contribution of the viscera, such as liver, intestine, stomach, spleen and pancreas in NST. Depocas (1958, 1960 a) functionally eviscerated rats by preventing blood flow through these organs. By demonstrating that noradrenaline was as effective in raising the oxygen consumption of functionally eviscerated cold-acclimated rats as it was in sham-operated rats, he was able to conclude that the viscera were probably not significantly important sites of NST.

The second approach has proved to be too indirect and conclusions concerning the potential involvement of various organs using their respective cytochrome oxidase activity are highly speculative.

Blood flow measurements have provided the most reliable information concerning tissue involvement in NST. Jansky (1971) stated the major problem of this approach:

"The best and ideal technique would be one based on organ blood flow and on extraction of oxygen from the blood. However, this represents a great obstacle in small animals where cannulation of small vessels under physiological conditions is pratically impossible."

Because of this problem indirect methods of measuring blood flow had to be used. The approach taken is to measure the cardiac output and its fractional distribution to individual tissues. These measurements allow calculation of tissue

blood flow. The maximum possible O_2 consumption of a tissue, and thus its potential thermogenic activity, can be determined from the calculated blood flow together with the measurement of the concentration of O_2 in arterial blood. Where it is possible to measure arteriovenous differences in oxygen across a tissue, the actual O_2 consumption of the tissue can also be obtained. There are basically two ways for estimating fractional distribution of cardiac output. Early studies used the Sapirstein technique (Sapirstein, 1958) which uses uptake of $^{86}Rb^+$ as an index of the distribution of cardiac output. Results of these studies showed that skeletal muscle contributes about 50% and brown adipose tissue less than 10% to the calorigenic response to NA (Jansky and Hart, 1968; Kuroshima et al., 1967; Evonuk and Hannon, 1963).

However, more recent work (Alexander et al., 1973; Foster and Frydman, 1978 a) has shown that the use of $^{86}Rb^+$ as a quantitative indicator of capillary flow is unreliable for a number of tissues, particularly for brown tissue, and that the microsphere technique should be the method used.

Alexander et al. (1972 & 1973) measured blood flow to various organs in young lambs using the rubidium and microsphere techniques respectively. Table II shows the differences in blood flow values obtained for brown adipose tissue. They concluded that the microsphere technique was a more valid measurement because it relied on simple physical principles, whereas the rubidium method dependent on the assumption that

TABLE II

Comparison of the Sapirstein and Microsphere Techniques for Estimating Blood Flow Through Perirenal Brown Adipose Tissue of Thermoneutral and Cold-Exposed Young Lambs

	Blood Flow (ml/100 g tissue·min)	
Method	Thermoneutral	Cold-Exposed
Sapirstein	71	145
Microsphere	188	1125

The table compares the mean blood flow obtained using the Sapirstein and microsphere techniques to perirenal brown adipose tissue in young lambs under thermoneutral and cold-exposed conditions. Cardiac output was estimated by the dye dilution technique and also by the Fick method based on oxygen consumption and the oxygen content of arterial and mixed venous blood. The distribution of cardiac output was determined using either the Sapirstein technique (Alexander et al., 1972) or the microsphere technique (Alexander et al., 1973). The significance of differences comparing blood flow values obtained at thermoneutral conditions with those obtained under cold-exposed conditions were $P<0.005$ using the Sapirstein technique and $P<0.01$ using the microsphere technique.

the indicator extraction ratio of each tissue was essentially the same as the mean for the whole body. Since Sapirstein (1958) had shown that a high diffusion barrier in brain existed which gave exceedingly low blood flow values, the conclusions of Alexander et al., (1973) appear to be reasonable.

Foster and Frydman (1978 a) extended the study concerning the rubidium method and by comparing flows estimated with $^{86}Rb^{+}$ with flows estimated simultaneously with microspheres for BAT and other tissues they concluded that for some tissues, especially BAT, the Sapirstein method of assessing fractional distribution of cardiac output to the tissue gives highly erroneous values.

Since it was shown that the labelled microspheres did not appear to have the above mentioned disadvantage Foster and Frydman (1978 b) used this approach and re-evaluated the problem concerning organ contribution to NST in the cold-acclimated rat. In their re-evaluation they discovered that during NA-induced calorigenesis there was a very large increase in blood flow to BAT (Table III). Increased flow to BAT was accomplished partly through an increase in cardiac output and partly through a change in the distribution of cardiac output (Table III). Their results also indicated that IBAT exhibited a remarkable ability to extract O_2 from the blood flowing through it (Table IV). They concluded that BAT contributes 60% or more to the metabolic response of the cold-acclimated rat to NA.

TABLE III

The Fractional Distribution of Cardiac Output and the Blood Flow to Tissues of Barbital-Sedated Cold-Acclimated Rats at Rest or During NA-Induced Calorigenesis

	Percentage of CO			Blood Flow (ml/min)		
Tissue	At Rest	With NA	P	At Rest	With NA	P
BAT	2.6 ± 0.1	33.5 ± 1.5	<0.001	2.3 ± 0.2	57.2 ± 3.8	<0.001
Muscle	11.2 ± 0.5	8.4 ± 0.4	<0.05	9.9 ± 1.2	14.5 ± 1.2	<0.02

The effects on tissue blood flow of infusing barbital-sedated rats with NA at a dose sufficient to achieve a maximum calorigenic response in cold-acclimated rats is shown. Changes in tissue blood flow related to NA-induced calorigenesis were assessed from measurements of flow in rats at rest followed by measurements in the same rats infused with NA at a dose of 12.5 ng $g^{-0.74}$ min^{-1}.

(From Foster & Frydman, 1978 b)

TABLE IV

Concentration of Oxygen in the Arterial Blood and in the Venous Effluent from IBAT of Barbital-Sedated Cold-Acclimated Rats at Rest or Responding Calorigenically to NA

	ml of O_2 per 100 ml blood	
	At Rest	With NA
Arterial	16.8 ± 0.3	13.4 ± 0.3
Venous (Sulzer's vein)	12.7 ± 0.4	0.1 ± 0.04
A-V difference	4.1 ± 0.5	13.3 ± 0.3

Table IV gives the concentration of O_2 in arterial blood, its concentration in the venous effuent from IBAT, and the $(A-V)O_2$ across the IBAT of barbital-sedated cold-acclimated rats at rest and during NA-induced calorigenesis. The O_2 contents of samples of arterial blood and of venous blood were determined with a Natelson microgasometer.
(From Foster & Frydman, 1978 b)

In conclusion, BAT is the major site of NST, regardless of its small size. The anatomical and physiological characteristics of BAT which make it unique in its thermogenic capacity are reviewed in the next Section.

5. Brown Adipose Tissue

Brown adipose tissue appears in some 56 species of mammals distributed through at least six orders. It is generally more abundant in those species and under those conditions in which nonshivering thermogenesis occurs (Smith and Hôrwitz, 1969). In nonhibernators the tissue grows during acclimation to cold and the mitochondria become larger and more numerous (Suter, 1969). In hibernators, which appear always to have brown adipose tissue, the tissue undergoes less marked changes during acclimation to cold (Ahlabo and Barnard, 1974).

The characteristic brownish color of the tissue is partly due to the high content of respiratory chain pigments, notably cytochromes, and also to the rich network of blood capillaries (Flatmark and Pedersen, 1975).

There are several areas in which brown fat occurs regularly in the rat (Smith and Roberts, 1964). The tissue is located around major blood vessels and nerve tracts in the chest and neck areas. It is also close to the spinal cord all the way down to the kidneys. The tissue is richly vascularized and both blood vessels and cells are innervated by the sympathetic nervous system.

The anatomical site of interscapular BAT is of special

interest since its venous drainage, via Sulzer's vein, is short and in close contact with the spinal cord. It has been postulated (Smith and Horwitz, 1969) that it plays a role in the suppression of shivering since heat derived from the interscapular region could warm up certain spinal cord thermal receptors which in turn are responsible for the control of shivering.

Thus, brown fat provides an internal heating jacket that overlies parts of the systemic vasculature and on signal becomes an active metabolic heater applied directly to the flowing bloodstream as it passes to and from the cooler periphery.

When a warm-acclimated rat is transferred to the cold the brown adipose tissue undergoes several characteristic changes (Suter, 1969). Among the earliest changes observed in BAT of cold-exposed rats is hyperemia, which is present throughout the first 24 hours of cold exposure and disappears by day 4. Enhanced vascular permeability seems to be associated with it, leading to an increase in the water content of the tissue. Lipid droplets diminish in size and small droplets are formed. These droplets are believed to fuse together and form the larger lipid droplets which are observed after longer exposure and in fully cold-acclimated rats. Hypertrophy and hyperplasia of the tissue occur, apparently due to proliferation and differentiation of precursor cells. There is also extensive growth and development of mitochondria.

An increase in mitochondrial volume develops early and persists as long as the animal remains in the cold. Mitochondrial cristae are remodeled first into a more irregular pattern composed of semicircles and loops. Subsequently, the cristae become straight and also more numerous.

Thus, as BAT grows in response to cold so too do the mitochondria. They become larger and more numerous per cell. They have more tightly packed cristae and hence have a greater respiratory capacity than mitochondria obtained from other tissues (Flatmark and Pedersen, 1975).

6. Biochemical Mechanisms of Heat Production in Brown Adipose Tissue

In brown adipose tissue, as in other tissues heat production is the result of combustion of substrate molecules, i.e. of respiration. An increase in respiration results in an increase in heat production. Current concepts of the regulation of respiration in mitochondria are based upon Michell's chemiosmotic hypothesis which proposes that the respiratory chain (Fig. 5 a) and the mitochondrial ATP synthetase (Fig. 5 c) are linked by a circuit of protons, the proton electrochemical gradient generated by the expulsion of protons from the mitochondrial matrix being utilized to drive the synthesis of ATP. If the sole pathway for the protons to re-enter the matrix is via ATP synthetase, linked stoichiometrically to the production of ATP and controlled by availability of the substrates, ADP and Pi, then when ATP synthesis

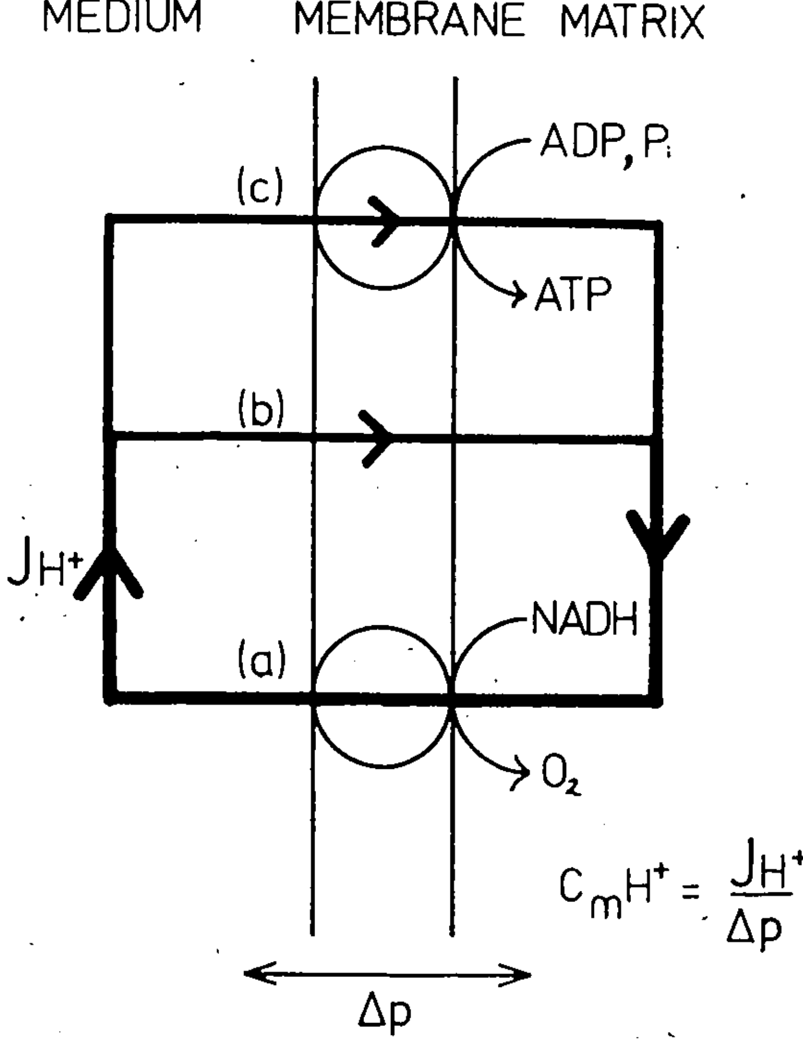

Figure 5. The Proton Circuit Across the Inner Membrane of Respiring Mitochondria

The figure displays the proton circuit proposed by the chemiosmotic theory to function during steady-state respiration. (a) Respiratory chain. (b) Proton leakage pathway. (c) Proton translocating ATP synthetase. JH^+: proton current, Δp: proton electrochemical gradient, C_mH^+: effective proton conductance of the membrane.

(From Nicholls, 1976 a)

ceases proton re-entry in turn ceases. This is turn rêsults in a build-up in the proton electrochemical gradient, stopping further proton release and hence respiration by the respiratory chain (Fig. 5 a & c).

From the above description, it can be seen that there are two ways in which heat production can be increased.

1) Decrease the phosphorylation state ratio (Owen and Wilson, 1974; Wilson et al., 1973).

Shivering thermogenesis is a good example of this type of thermogenic process. Muscle contractions utilize ATP and thus provide ADP which permits the acceleration of mitochondrial respiration.

2) By allowing protons to re-enter the matrix by an aternative pathway not linked to the synthesis of ATP (Fig. 5 b). An example of this is DNP-induced stimulation of respiration whose uncoupling action is believed to be due to its ability to transport protons across the membrane, thus short-circuiting the proton current (Mitchell, 1976).

The above mentioned mechanisms introduce the two major theories concerning the mechanism of NA stimulated heat production in BAT. The first theory proposes that increased pumping of Na^+ and K^+ by the Na^+-K^+ ATPase is responsible for a significant part of the increase in metabolic rate.

The second theory proposes that mitochondria obtained from BAT are loosely coupled and therefore their respiration is no longer controlled by the availability of ADP.

a) The Na^+ K^+-ATPase Mechanism

A role in BAT for the Na^+ K^+-ATPase derives from Edelman's hypothesis concerning its contribution to heat production in response to thyroid hormone (Edelman, 1974). This hypothesis is applicable to BAT since it has been shown that NA depolarizes this tissue (Williams and Matthews, 1974), increases membrane permeability to ions (Horowitz et al., 1971) and causes efflux of K^+ and influx of Na^+ (Girardier and Seydoux, 1971). In addition to allowing distribution of ions which would by itself stimulate the Na^+ pump, NA and cAMP have been shown to directly stimulate Na^+ K^+-ATPase activity in homogenates of BAT (Herd et al., 1970; Horwitz & Eaton 1975).

Seydoux et al, (1977) and Seydoux and Girardier (1978) have determined the temporal sequence of events induced by stimulation of the nerve supply of brown adipose tissue. Three events were studied;

a) membrane potential which reflects transmembrane ionic movements.

b) pyridine nucleotide redox state which indirectly reveals the activation of the adenylate cyclase system and subsequent lipolysis and mitochondrial events.

c) intratissue PO_2 tension which reflects the overall thermogenic response of the tissue,

They noted at stimulation frequencies above 3 Hz*, the

*The response of brown fat is a function of the number of nerve impulses delivered per unit time and not of the number of nerve fibers activated.

first event to occur was the depolarization of the cell membrane, then the increase in NAD(P)H and finally, the increase in cell respiration.

While the above mentioned evidence would appear to indicate that an ATPase type of mechanism could play a potentially significant role in producing heat it is unlikely that this type of mechanism makes more than a minor contribution. While Seydoux et al. (1977) have determined the temporal sequence of the events induced by stimulation of the nerve supply to brown adipose tissue they also discovered that if stimulation frequencies below 3 Hz were applied no depolarization whatsoever occurred even though there was an increase in metabolic activity. This then indicates that depolarization is not absolutely required for the thermogenic response to occur.

The capacity of IBAT mitochondria for ATP synthesis is rather low, as indicated by their low ATPase activity (Cannon & Vogel, 1977) and by the absence of the characteristic protrusions on the inner surface of the inner membranes, believed to correspond to the ATPase complex (Lindberg et al., 1967). Translocation of ADP and ATP into and out of BAT mitochondria is highly sensitive to inhibition by acyl·CoA and would appear to have limited activity _in vivo_ during NA-induced thermogenesis (Christiansen et al., 1973). Thus it is unlikely that an ATPase mechanism, which would require extensive rephosphorylation of ADP, could be a major thermogenic process during nonshivering thermogenesis.

Since BAT mitochondria appear to become loosely-coupled during nonshivering thermogenesis (see (b) below), an alternate source of ATP for the operation of the Na^+ K^+-ATPase is necessary. A substantial amount of ATP can be formed in the mitochondrion by substrate level phosphorylation (Rafael et al., 1974; Nicholls & Bernson, 1977) derived from the tricarboxylic acid cycle which is accelerated under thermogenic conditions and hence this process could provide a source of the ATP required by the Na^+ K^+-ATPase.

In conclusion, while it is known that an ATPase type of mechanism does contribute to the calorigenic response to NA in BAT, its relative contribution would appear to be small.

b) Loose-Coupling Mechanism

The second theory, which applies to BAT mitochondria, is that they are loosely coupled. There are two ways in which this can be achieved. 1. A loose-coupling mechanism can occur when the cycling of ions is promoted. If the uptake of the ion is energy-dependent and if the ion is allowed to leak out, thus making it available for active transport, then this would make the mitochondria loosely-coupled. An example of this is calcium transport. This type of loose-coupling mechanism will be examined further in the next section. 2. A loose-coupling mechanism can occur if protons are allowed to re-enter by a proton leakage pathway. The evidence in favor of this type of mechanism will now be discussed.

BAT isolated by conventional methods are usually uncoupled

or loosely coupled. This is indicated by their rapid rates of respiration, low P/O ratios and lack of respiratory control (Flatmark and Pedersen, 1975). Because recoupling requires treatment with ATP and carnitine or with albumin (Flatmark, & Pedersen, 1975) it has been generally concluded that removal or combustion of a small proportion of the fatty acids bound to the mitochondria is necessary. It therefore appears that free fatty acids play a dual role in BAT mitochondria; fuel as well as uncoupling agent (Bulychev et al., 1972). In addition, it has been shown that a purine nucleotide (eg. ADP, ATP, GDP, GTP) is also necessary for recoupling (Cannon et al., 1973). The importance of these added nucleotides is shown by the fact that in their absence even exhaustive removal of endogenous fatty acids does not recouple the mitochondria and the mitochondria still have a rapid uncontrolled respiration without synthesizing ATP. Addition of purine nucleotides to the medium in low concentrations produces a transformation into classic respiration-controlled mitochondria. Until recently, the action of these added nucleotides was not understood. In 1976 Nicholls described a purine nucleotide binding site present in BAT mitochondria and absent from liver mitochondria. It was suggested that occupation of this site, which was shown to be located on the outer surface of the inner membrane, by added purine nucleotides inhibited proton ion conductance which was noted to be high in freshly isolated mitochondria. This site has recently been identified as

involving a polypeptide of molecular weight 32,000 and acccounts for 10% of the inner membrane of hamster brown adipose tissue mitochondria (Heaton et al., 1978).

In other words, brown adipose tissue mitochondria possess a proton conductance pathway which enables protons to leak back across the inner membrane thereby lowering the proton electrochemical gradient (Figure 5 b) and stimulating respiration. It is clear that the rate at which protons are expelled by the respiratory chain must equal the rate at which they re-enter the matrix either via the proton translocating ATP synthetase (c in Figure 5) or via the 32,000 polypeptide pathway (b in Figure 5). If the conductance of the two pathways is inadequate, the difference in the proton electrochemical gradient will build up and respiration will be inhibited (see Nicholls, 1977 for review).

That the purine nucleotide binding is related to the thermogenic capacity of a tissue is shown by several findings. Nucleotide binding is high in newborn guinea pigs and decreases with age (Rafael & Heldt, 1976). It is also high in the cold-acclimated rat (Desautels et al., 1978; Sundin & Cannon, 1978) and high in the cold-acclimated hamster (Nicholls, 1976 b). The amount of the 32,000 polypeptide is also high in cold-acclimated rats (Desautels et al., 1978), guinea pigs and hamsters (Heaton et al., 1978).

Desautels et al. (1978) have studied the adaptive changes in purine nucleotide binding by following the increases in binding of ADP and GDP and in amount of the 32,000 polypeptide

as a function of cold exposure. As can be seen (Figure 6) there is an increase in binding of both GDP and ADP which is apparent within an hour and reaches a maximum in 3-7 days. However, the amount of the 32,000 polypeptide only begins to increase at 12 hours and reaches a maximum only after two weeks (Fig. 7). These results suggest that there is an initial unmasking of sites before the amount of the 32,000 polypeptide increases.

The presence of a proton leakage pathway in BAT mitochondria which can be made open or closed provides a mechanism for physiological uncoupling. However, the nature of the messenger which is capable of switching on and off the proton conductance is not known. The fact that purine nucleotides themselves do not appear to change extensively or rapidly enough on addition of NA indicates that it is improbable that they are the messenger (Pettersen & Vallin, 1976). Cannon et al. (1977) have suggested that acyl CoA formed as a result of NA-induced lipolysis and subsequent activation of fatty acids could displace the bound nucleotides and hence cause a loosening of coupling in these mitochondria.

Although all the events of NA-induced thermogenesis in BAT have not been deduced the following is a mechanism which attempts to summarize the present concepts of NST (Figure 8).

On stimulation of the sympathetic nerves, NA is released and binds to a receptor on the plasma membrane which causes stimulation of adenylate cyclase to produce cAMP. Depolari-

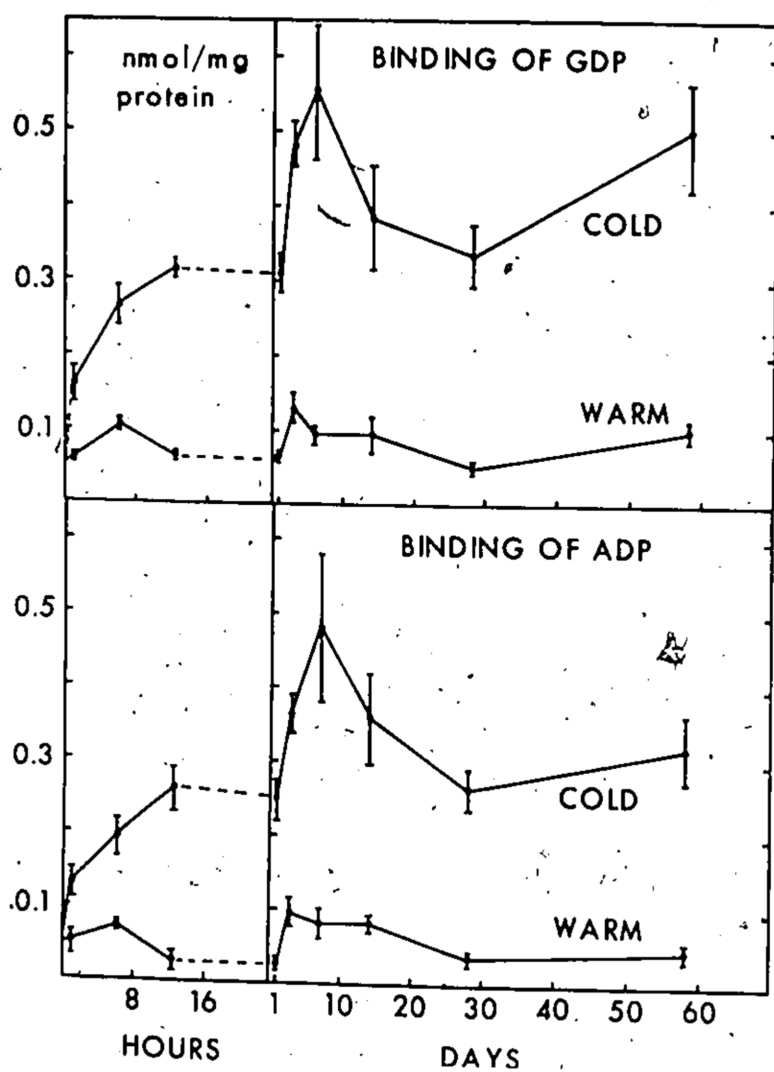

Fig. 6. Binding of GDP and ADP by Brown Adipose Tissue Mitochondria During Acclimation to Cold.

The graphs compare the binding during acclimation to cold (COLD) with the binding in control rats (WARM). The left-hand part of each section of the figure shows changes during the first day and the right-hand part shows changes during the subsequent 8 weeks. Values are means (± SEM) of three observations.

(From Desautels et al., 1978)

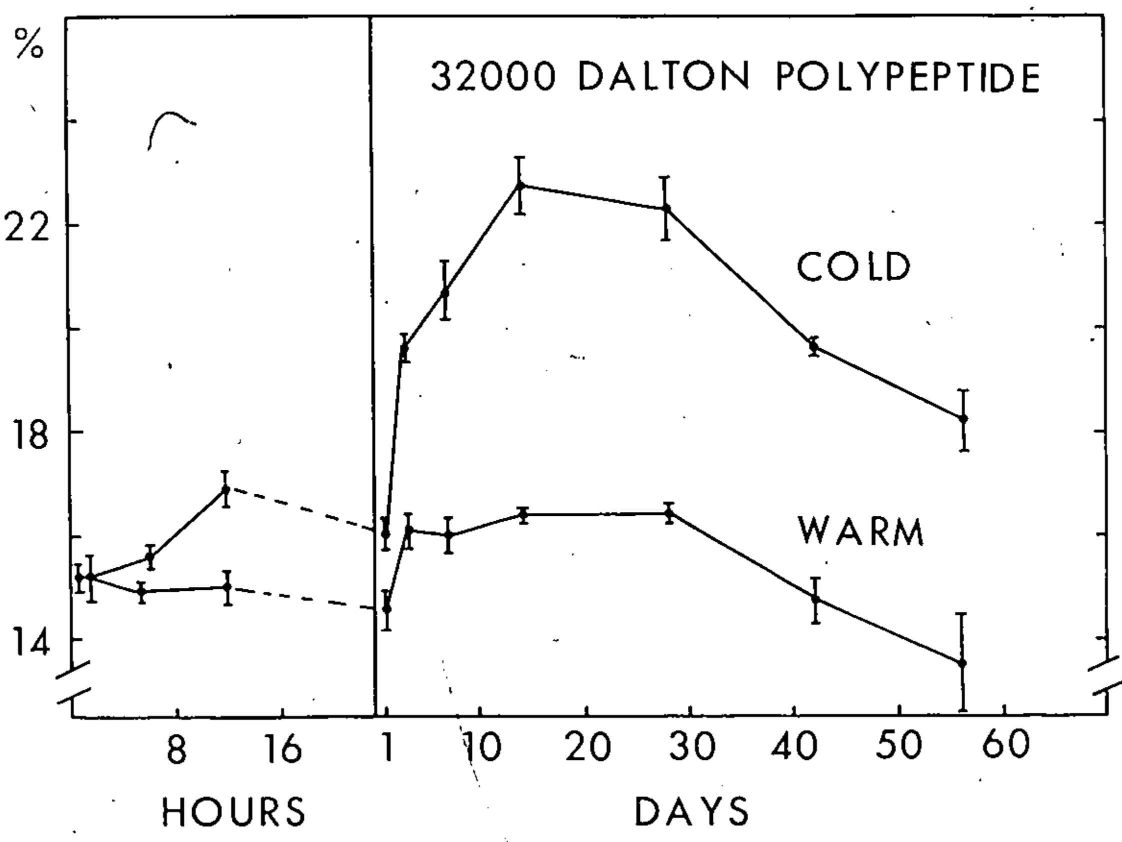

Figure 7. Proportions of the 32,000 Polypeptide of Brown Adipose Tissue Mitochondrial Membrane Protein During Acclimation of Rats to Cold.

The figure shows the increase in the proportion of the 32,000 polypeptide to cold. For each point, the number of rats studied is two or three. Note that the scale on the x-axis does not start at zero. The time scale is expanded in the left-hand portion of the figure to illustrate events during the first 24 h.

(From Desautels et al., 1978)

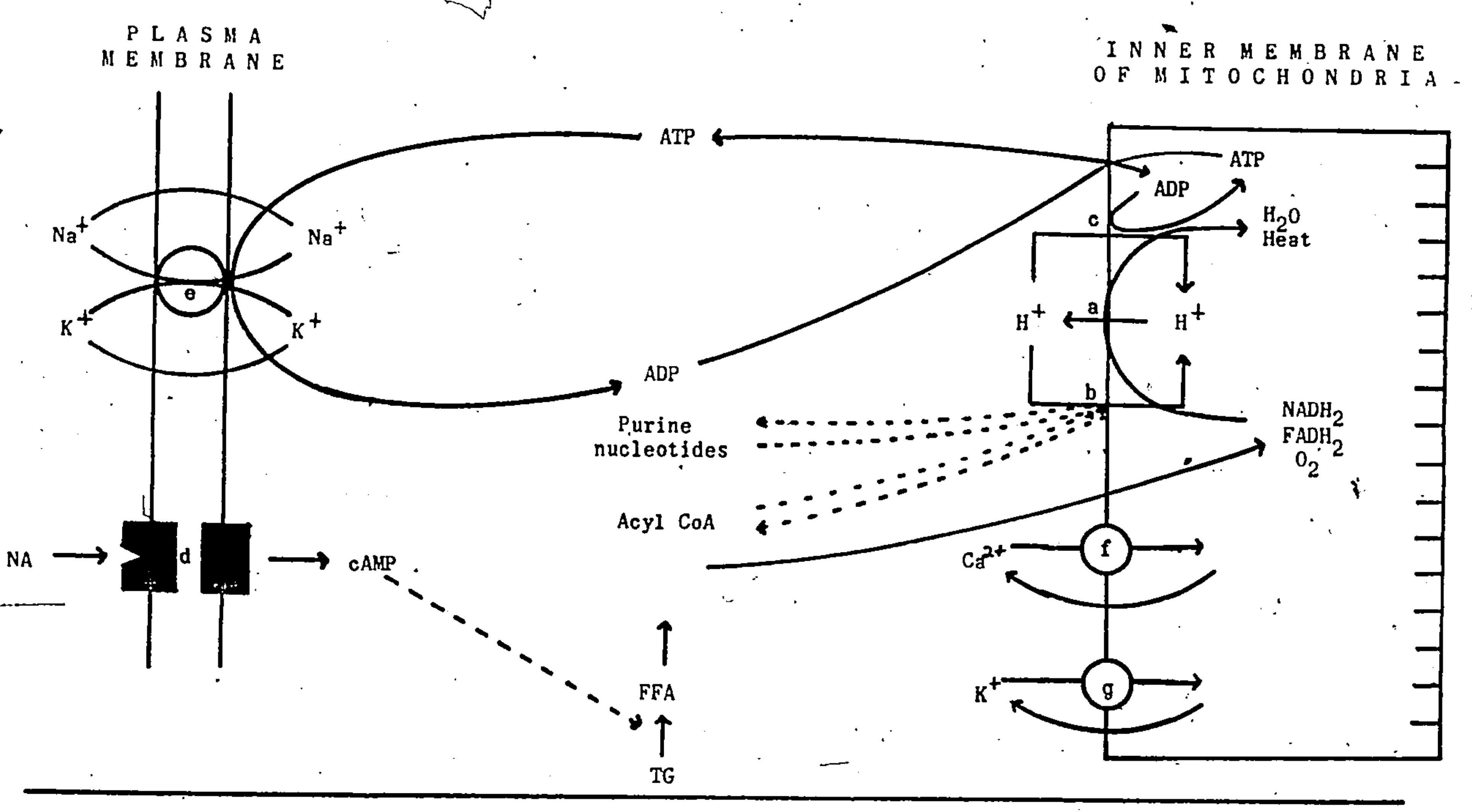

Figure 8. Suggested Mechanisms for Nonshivering Thermogenesis in Brown Adipose Tissue

The figure summarizes the sequence of events believed to occur during noradrenaline-induced thermogenesis in brown adipose tissue. (a) Respiratory chain. (b) Proton conductance pathway, site of the 32,000 molecular weight protein. (c) Proton translocating ATP synthetase. (d) Noradrenaline receptor responsible for the stimulation of adenylate cyclase and depolarization of the membrane. (e) The Na^+K^+ATPase. (f) Calcium uptake and release. (g) Potassium uptake and release.

zation of the membrane may also occur. cAMP activates a triglyceride lipase and thus causes increased lipolysis to occur. The fatty acids so liberated are activated to fatty acyl CoA which displaces purine nucleotides from their binding sites on the 32,000 polypeptide of the proton conductance mechanism and makes the mitochondria loosely coupled by increasing proton conductance. The end result is to stimulate respiration and produce heat which warms the blood flowing through the tissue.

PART B: IONS IN MITOCHONDRIA AND THE REGULATION OF CELLULAR METABOLISM

1. Introduction

As we have seen, evidence has favoured a loose-coupling mechanism, rather than an ATPase type of mechanism, to explain the high thermogenic capacity of brown adipose tissue. Loose-coupling mechanisms can be further classified into two types: I) one in which the usual impermeability to protons of the inner mitochondrial membrane is specifically disrupted (as described in Section 6(b) above) II) one in which the cycling of other ions is promoted. In the latter type of mechanism an apparent uncoupling occurs when the electrochemical gradient is utilized for ion transport. The purpose of this review is to introduce the concept of ion transport as a contributing factor in the control of respiration in mitochondria and to indicate how such a mechanism might be regulated.

2. Mitochondrial Ions and Their Distribution

Before discussing how metal ions could play a role in energy metabolism, and hence thermogenesis, it is necessary to review the contents and distribution of metal ions in the mitochondrion. Table V shows a summary of the content of various metal ions in mitochondria from several different tissues. As can be noted, the level of K^+ is usually quite high in isolated mitochondria. The low K^+ level of brown adipose tissue mitochondria can be explained by the high permeability to mono-

TABLE V

Ionic Contents of Mitochondria

Tissue	Ion	Ion Contents (nmoles/mg protein) Warm-Acclimated	Cold-Acclimated	Reference
Rat Liver	K^{+}	170.0	155.0	Bramante & Nirdlinger, 1974
	Ca^{2+}	4.0	6.0	McBurney & Radomski, 1973
		6.0		Bramante & Nirdlinger, 1974
		10.04-14.0		Carafoli & Lehninger, 1971
	Mg^{2+}	43.0	42.0	McBurney Radomski, 1973
		45.0		Bramante & Nirdlinger, 1974
		19.0		Hoser et al., 1976
		25.0		Siliprandi et al., 1975
		23.0		Bogucka & Wojtczak, 1971
Rat Heart	K^{+}	165.0	173.0	Bramante & Nirdlinger, 1974
		99.0		Settlemire et al., 1968
	Ca^{2+}	30.0-40.0		Carafoli & Lehninger, 1971
		5.7	5.6	Bramante & Nirdlinger, 1974
	Mg^{2+}	23.0		Settlemire et al., 1968
		35.0	40.0	Bramante & Nirdlinger, 1974
		22.0		Bogucka & Wojtczak, 1971
Rat Muscle	Ca^{2+}	15.0	15.0	Greenway & Himms-Hagen, 1978
		28.0		Thakar et al., 1973
	Mg^{2+}	34.0		Thakar et al., 1973
Rat BAT	Ca^{2+}	44.5	20.0	Greenway & Himms-Hagen, 1978
	K^{+}	9.2		Drahota, 1970
Guinea Pig BAT	Ca^{2+}	81.0	12.2	Christiansen, 1971

valent cations of the freshly isolated mitochondria (Nicholls, et al., 1972).

K^+ appears to serve as the principal cation for numerous anions of metabolic importance, such as the tricarboxylic acid cycle intermediates, phosphate and the adenine nucleotides which are formed in the matrix compartment of isolated mitochondria (Brierly, 1976). The K^+ of mitochondria can be divided into three components, a rapidly exchangeable component is thought to represent cation bound on the membrane surface, a rapidly removable component which represents soluble K^+ sequestered in the intermembrane space, and a component which exchanges only slowly and is retained on washing. This component is considered to be K^+ sequestered or bound in the matrix compartment (Brierley, 1976).

The calcium content of mitochondria obtained from a variety of tissues is generally in the range of 5-15 nmoles/mg mitochondrial protein. Brown adipose tissue mitochondria are an exception having calcium contents much higher than other tissues (Christiansen, 1971; Greenway & Himms-Hagen, 1978). Calcium ions can interact with the mitochondrion in one of three ways (Binet & Volfin, 1975).

I) They can regulate or interfere with many mitochondrial functions. They do this by binding to specific regulatory binding sites which may either be located on the membrane or in soluble form.

II) They can bind to structural sites and hence be implicated in electrostatic binding between negative charges of membrane proteins and phospholipids.

III) They can bind to a specific calcium carrier.

As shown in Table V, Mg^{2+} is usually more abundant in the mitochondrion than Ca^{2+}. It too can interact with the mitochondrion in much same way as Ca^{2+} with the possible exception of binding to carrier sites (Brierley, 1976). Table VI indicates that significant portions of endogenous Mg^{2+} are found in the intermembrane and matrix compartments (Bogucka & Wojtczak, 1971). Since the intermembrane space contributes only a small portion of the total mitochondrial volume and contains a small percentage of mitochondrial protein, the concentration of magnesium in this space must be very high. The finding that the outer membrane is permeable to Mg^{2+} (O'Brien & Brierley, 1965) and that washing liver mitochondria in isotonic sucrose containing EDTA does not appreciably diminish the magnesium content in either compartment (Bogucka & Wojtczak, 1971) suggests that the magnesium present in the intermembrane space is in a bound, non-diffusible form. Bogucka and Wojtczak (1976) provided further evidence supporting this suggestion by demonstrating the presence of at least two distinct high molecular weight compounds, located in the intermembrane compartment, capable of binding magnesium. The higher molecular weight component was found to have a lower affinity, but a higher binding capacity for magnesium than the lower molecular weight component.

TABLE VI

Distribution of Magnesium in Mitochondria from Rat Liver and Heart

Tissue	Outer Membranes	Intermembrane Compartment	Inner Membranes	Matrix	Total nmoles/mg protein
Liver	4%	50%	5%	41%	22.0
Heart	5%	31%	21%	43%	17.6

(From Bogucka & Wojtczak, 1971)

Neither the identification nor the role of these magnesium binding proteins is understood. The inner membrane and matrix compartment of rat liver mitochondria contain approximately half of the mitochondrial magnesium, i.e. about 10 nmoles/mg mitochondrial protein. Since this is approximately equal to the amount of mitochondrial adenine nucleotides, which is also contained in the matrix compartment (Ernster et al., 1967), it supports the assumption that most of the mitochondrial adenine nucleotides are present as magnesium complexes.

3. Ion Transport in Mitochondria

(a) Introduction

According to the chemiosmotic theory, the proton gradient established by oxidative electron transport represents a store of free energy. The energy is stored in the proton gradient in two forms, or in other words, the gradient has two components. One component is the difference in concentration or chemical activity of protons on opposite sides of the membrane. The energy of the concentration gradient is determined by the difference in pH across the membrane (ie. Δ pH). The electric charge carried by the proton contributes the second component of the energy of the gradient. The net movement of charge across the membrane creates a difference in electric potential, and all charged particles are affected by the resulting electrostatic field (ie. $\Delta \Psi$). The total energy of the proton gradient is the sum of the concentration component and the electric component.

Therefore, a proton which has been expelled from a mitochondrion experiences a force tending to draw it back across the membrane. The movement of the proton to that force can be made to do work, such as actively transporting certain ions. Experimentally, the transport of ions across the mitochondrial inner membrane is usually revealed by osmotic swelling and contraction techniques (Brierley, 1974). Osmotic swelling in the presence of electrolytes will occur only when both an anion and a cation can enter the matrix compartment so that the internal osmolarity can be increased without inducing a large diffusion potential (Chappel & Crofts, 1966). Large accumulations have been observed when either an insoluble salt, such as calcium phosphate, is formed which removes the accumulated solutes from solution, or a soluble anion-cation, such as potassium acetate, is taken up under conditions which permit osmotic swelling of mitochondria.

This review will be mainly concerned with the transport of cations across the mitochondrial inner membrane. The necessary accumulation of anions in order to obtain swelling would appear to limit the usefulness of osmotic swelling as an assessment of the entry mechanism of a given cation. However, by interchanging various anions in the suspending medium and by using ionophores* of established properties it has been

*Ionophores are molecules that carry ions across membranes. The ionophore valinomycin is a cyclic molecule that is soluble in membranes and selectively carries potassium ions. Nigericin also transports potassium, but only in exchange for protons. Gramicidin A forms a pore in the membrane, made up of two helical molecules, that is permeable to ions with one positive charge.

possible to obtain useful information.

Before discussing the characteristics of cation transport it is necessary to review the different types of anions used experimentally and their known mode of entry into the mitochondrion. There are three fundamental mechanisms for anion penetration in nonrespiring mitochondria (Fig. 9).

1) anion exchange. Fig. 9 shows that $H_2PO_4^-$ exchanges for ^-OH on the phosphate transporter. This reaction is equivalent to the entry of undissociated H_3PO_4 followed by a loss of a proton to the alkaline matrix.

2) electrogenic anion penetration. Several anions, such as nitrate and thiocyanate appear to diffuse across the membrane at neutral pH in response to either a chemical or electrical gradient.

3) free acid penetration. Most weak acids, such as acetic acid penetrate the membrane undissociated. Ionization in the interior results in H^+ release.

(b) Characteristics of Monovalent Ion Uptake

Nonrespiring mitochondria suspended in acetate or phosphate salts of K^+ do not swell, while mitochondria suspended in Na^+ salts undergo rapid spontaneous swelling. This has been attributed to the presence of an electrically neutral exhanger specific for Na^+ and H^+ which is present in the inner membrane (Fig. 10, Brierley, 1974). Passive swelling, i.e. swelling in the absence of respiration, can be induced in mitochondria suspended in K^+ salts by the use of reagents, such as ionophores, which increase the permeability of the membrane to monovalent

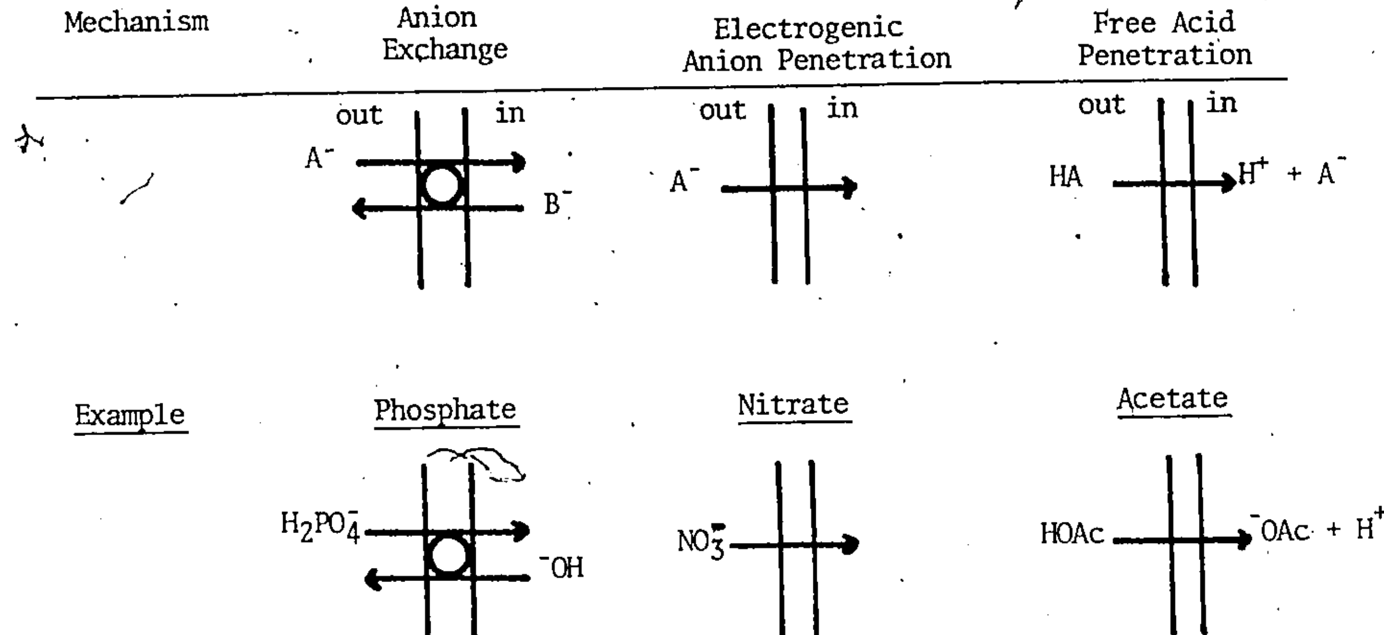

Figure 9. Pathways for Penetration of Anions into Mitochondria in the Absence of Metabolic Energy

See text for details

(From Brierley, 1974)

Mechanism	Electrically Neutral Exchange	Electrogenic Cation Penetration
	Na+ / H+	K+ / val / K+
Example	Na^+	K^+-valinomycin
Passive swelling: Nitrate Salt	Requires uncoupler	Requires val
Passive swelling: Acetate Salt	Spontaneous	Requires (val + uncoupler) or nigericin

Figure 10. Pathways for Penetration of Cations into Mitochondria in the Absence of Metabolic Energy

See text for details.

(From Brierley, 1974)

cations. Passive swelling in K^+ acetate requires not only a pathway for K^+ entrance, but also a pathway for the removal of the internal H^+ accumulation generated by the entry of acetic acid (see Fig. 9). A pathway for K^+ influx and H^+ efflux can be achieved simultaneously by the use of nigericin, which induces K^+/H^+ exchange, or achieved separately by using the mobile cationophore valinomycin, which allows K^+ influx, in the presence of an uncoupler which allows H^+ efflux (Fig. 10, Brierley, 1974).

In contrast to the results for passive swelling, mitochondria suspended in acetate or phosphate salts of K^+ and other cations take up ions and swell extensively when the mitochondria are energized by addition of substrate or ATP. The reaction occurs in the absence of reagents which modify permeability, is sensitive to uncouplers, inhibitors of respiration, and to oligomycin when exogenous ATP is used as the energy source. Monovalent ion uptake is believed to occur via an electrophoretic pathway (Fig. 11). There is no clear cut evidence that the electrophoretic movement of K^+ or Na^+ is carrier mediated. The most widely accepted mechanism (Fig. 11) involves the use of the electrochemical gradient generated by respiration as the primary event. Phosphate and acetate are pulled into the alkaline matrix of respiring mitochondria because of their capacity to donate protons. Their entry thus converts a portion of the Δ pH to a phosphate and acetate gradient respectively (i.e. $\Delta \Psi$ is increased). Electrogenic cation flow follows sufficient

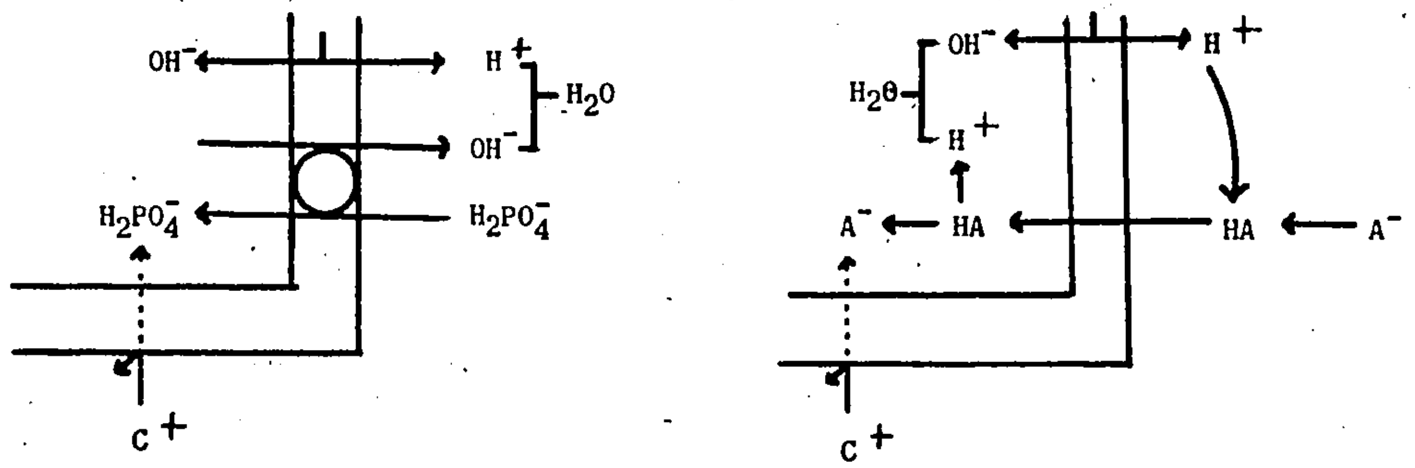

Figure 11. Electrogenic Uptake of Monovalent Cations

Electron transport generates a pH gradient across the membrane. This is followed by the exchange of anions for internal OH^-. Thus, $H_2PO_4^-$ (left figure) may exchange for matrix hydroxide ions on the phosphate-hydroxide antiporter, a reaction formally equivalent to the entry of undissociated H_3PO_4, followed by loss of a proton to the alkaline matrix. Salts of weak acids, such as acetate (right figure), are pulled into the alkaline matrix because they actually pass the membrane as the corresponding free acids which then yield their protons to the excess ^-OH in the matrix. The net effect of the entry of these anions into the matrix of respiring mitochondria is to convert the alkaline-inside electrochemical gradient of protons generated by electron transport into a negative-inside gradient of the transported anion. The normal impermeability of the membrane to cations would be overcome by this negative interior potential and cation penetration would then result in K^+ acetate (or phosphate) accumulation and osmotic swelling.

(Adapted from Chavez et al., 1977)

driving potential builds up. It is implicit in this model that the process is limited by the low permeability of the membrane to cations under normal conditions. There are, therefore, two ways in which monovalent cation flow and hence swelling, can occur: 1) when the interior electric potential becomes large enough (i.e. by using acetate or phosphate salts) to overcome the permeability barrier or 2) by increasing the permeability of the membrane to monovalent cations by using ionophores. In addition to increasing the passive permeability to cations the use of ionophores also markedly activates the energy - dependent ion accumulation reaction. The most rapid extensive energy - dependent swelling occurs with K^+ salts (Brierley et al., 1971).

As Fig. 11 shows, K^+ uptake is driven by only one component of the electrochemical gradient, namely the electrical component (i.e. $\Delta \Psi$). This is because the transport of a monovalent cation is entirely motivated by electrostatic forces that pull it toward the negatively charged inner surface of the membrane and hence net accumulation of cations will occur when $\Delta \Psi$ is large compared to Δ pH. Isotonic salts of acetate or phosphate are generally used for ion uptake experiments because these compounds are capable of converting the Δ pH component of the electrochemical gradient into an acetate or phosphate (i.e. $\Delta \Psi$) gradient respectively.

Mitochondria swollen in Na^+ or K^+ nitrate are able to contract and extrude the accumulated salt in a respiration - de-

pendent reaction. The contraction reaction is characterized by high rates of respiration which return to normal when ion extrusion is complete (Brierley, 1976; Brierley et al., 1977). It is believed that monovalent cation extrusion occurs via an exchange pathway (Fig. 12). The reaction is more rapid and extensive in Na^+ than in K^+ nitrate.

In the case of the efflux reaction, the difference in proton concentration, Δ pH, is the only driving force. The membrane potential has no effect since while it accelerates the proton's inward passage it retards the outward movement of the monovalent cation and the two effects exactly cancel. The reason why most contraction studies are done in salts of nitrate is due to the ability of the permeable NO_3^- ion to dissipate the electrical component of the electrochemical gradient thus leaving a large Δ pH which is required for the contraction process. A model for monovalent cation uptake and extrusion using K^+ as an example is shown in Fig. 13 (Chavez et al., 1977). As shown, respiration produces a Δ pH which can be partially converted to a $\Delta\ \Psi$ by acetate or phosphate. A voltage gated K^+ uniport permits electrophoretic K^+ entry when $\Delta\ \Psi$ exceeds a certain limiting value and a K^+/H^+ exchanger comes into play when Δ pH attains sufficient magnitude.

The presence of both a monovalent uniport and a monovalent/H^+ exchanger would imply that some form of regulatory mechanism be present to prevent futile cycling of these ions. It can therefore be speculated that removal of this regulatory mechanism

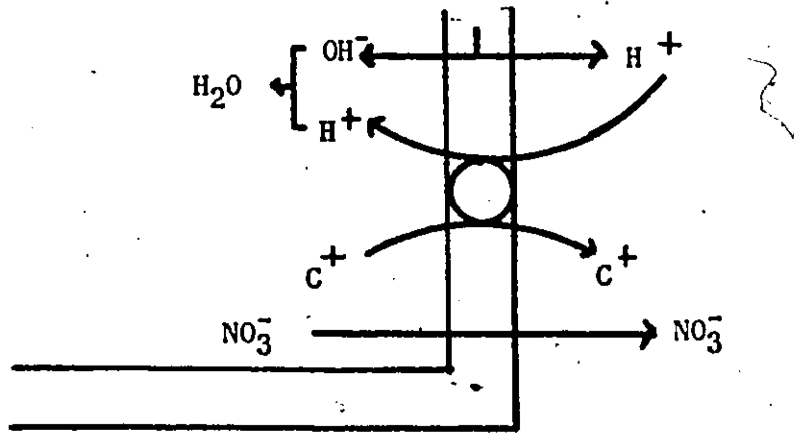

Figure 12. Exchange Mediated Extrusion of Monovalent Cations

Respiration produces a pH gradient and the resulting exterior H^+ is able to exchange for interior C^+ via the mitochondrial cation/H^+ exchanger. The entry of H^+ by the exchanger produces water in the interior with a decrease in net positive charge causing an electrophoretic anion efflux which results in osmotic contraction. This mechanism assumes that the rate of cation extrusion on the exchanger is greater than the rate of cation leak into the matrix, that electrophoretic H^+ permeability is low, and that there is sufficient permeability to internal anions to permit their electrophoretic exit. $C^+ = Na^+$ or K^+.

(From Brierley, 1976)

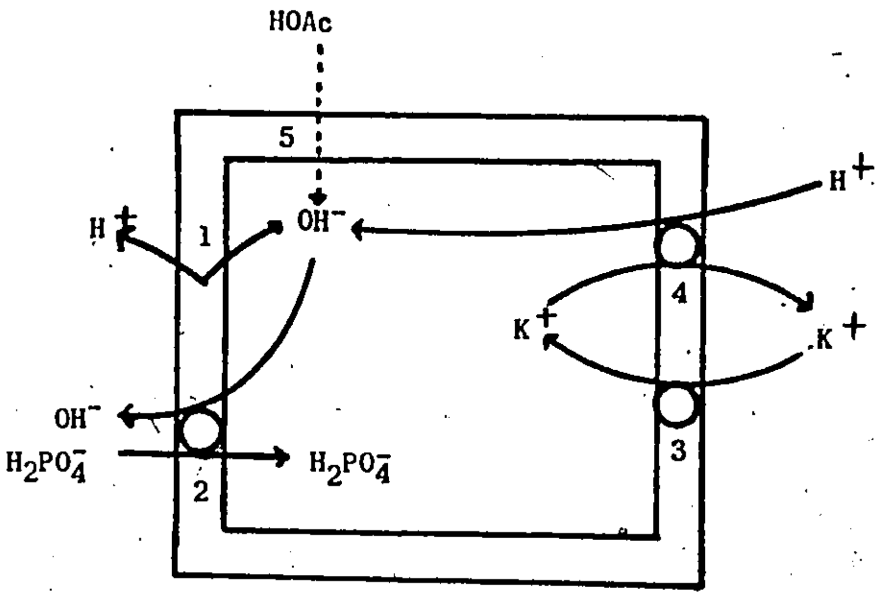

Figure 13. Proposed Model for K^+ Influx and Efflux in Mitochondria

The pH gradient generated by respiration (1) results in the entry of phosphate (2) or acetate (5). The entry of these ions converts a portion of the Δ pH to $\Delta \Psi$. A voltage-gated K^+ uniport (3) permits electrophoretic K^+ entry when $\Delta \Psi$ exceeds a certain limiting value, and a K^+/H^+ exchanger (4) comes into play when Δ pH attains sufficient magnitude.

(From Chavez et al., 1977)

could provide a thermogenic mechanism since both the monovalent uniport and the monovalent/H^+ exchanger would stimulate respiration which would result in an increase in heat production. In this type of thermogenic mechanism respiration would no longer be controlled by the availability of ADP.

(c) Characteristics of Divalent Ion Uptake

(1) Calcium Transport

Mitochondria isolated from all major tissues including liver, kidney, heart, brain and spleen have the capacity to accumulate Ca^{2+} at the expense of the energy of electron transport or of ATP (Carafoli & Lehninger, 1971). That the transport is carrier mediated is supported by several findings. Calcium transport has the properties of substrate saturability (Bygrave et al., 1971; Spencer & Bygrave, 1973). The fact that graphs of Ca^{2+} accumulation as a function of increasing concentrations of added Ca^{2+} are sigmoidal rather than hyperbolic indicates cooperativity. The affinity of mitochondria for Ca^{2+} uptake is very high, with measured Km's ranging from less than 1 μM to about 5 μM (Reynafarje & Lehninger, 1969; Spencer & Bygrave, 1973). Calcium uptake is inhibited specifically by lanthanides (Mela, 1969) and by the histochemical stain ruthenium red (Moore, 1971) in micromolar concentrations. Mitochondria from yeast and blowfly flight muscle are unable to accumulate Ca^{2+} in a respiration-dependent process and possess no high-affinity Ca^{2+} binding sites. This finding indicates a genetic determination (Carafoli & Lehninger, 1971; Carafoli et al., 1971).

The transport of Ca^{2+} can be visualized as occuring in several steps (Bygrave, 1977).

1. Interaction at the outer surface of the inner membrane: Ca^{2+} binds with high affinity to two interacting binding sites for Ca^{2+}; these contain carboxyl groups and are situated on the carrier.

2. Translocation through the inner membrane: The bound Ca^{2+} is rapidly transported from the outside of the membrane to the inside of the membrane in response to the membrane potential. The process most likely involves conformational changes in the carrier molecule.

3. Release of the Ca^{2+} into the matrix space: The release of the transported Ca^{2+} into the matrix space is the rate-limiting step in the entire transport system. It is promoted by proton-yielding permeant anions, such as phosphate.

4. Presumably the carrier then reverts to its original state in order to transport further Ca^{2+} ions into the matrix space of the mitochondrion.

Since phosphate promotes the rate-limiting release of Ca^{2+} into the matrix its presence enables large quantities of Ca^{2+} (over 3000 nmoles Ca^{2+}/mg protein) to transported. In the absence of phosphate, however, the calcium taken up does not appear in the matrix but is bound to the outer surface of the inner membrane. This type of energy-dependent binding is limited to about 100 nmoles Ca^{2+}/mg protein and is called membrane loading. However these binding sites which are also located on specific proteins in the inner membrane are not those

of the Ca^{2+} carrier molecules. The addition of Ca^{2+} to respiring mitochondria stimulates respiration and for each pair of electrons passing each energy-conserving site of the respiratory chain, two calcium ions are accumulated. Simultaneously, two H^+ ions are ejected for each Ca^{2+} taken up (Brand et al., 1976). If a respiratory inhibitor is added, then the previously bound Ca^{2+} is immediately discharged with uptake of H^+.

A model for energized membrane loading has been presented by Lehninger (Fig. 14, 1972). He has proposed that the Ca^{2+} binding sites, which are responsible for membrane loading, are located on specific proteins in the inner membrane, independent of the respiratory chain and Ca^{2+} carrier molecules. These proteins are suggested to contain protonated groups exposed to the inner or matrix side of the inner membrane. In the absence of electron transport, these groups remain protonated and in this state the Ca^{2+} is bound with low affinity to the outer surface. When respiration is instituted, with build-up of hydroxyl on the inside, the protons on the inner surface of the binding proteins are removed to relieve the internal alkalinization. Simultaneously, the protein undergoes a conformational transition to a state capable of binding external Ca^{2+} with high affinity, a change comparable to the Bohr effect of hemoglobin, in which the degree of oxygenation is linked to the pH via conformational changes in the molecule. In this way respiration-linked membrane loading of Ca^{2+} may be coupled to electron transport and H^+ ejection. Uncoupling agents would

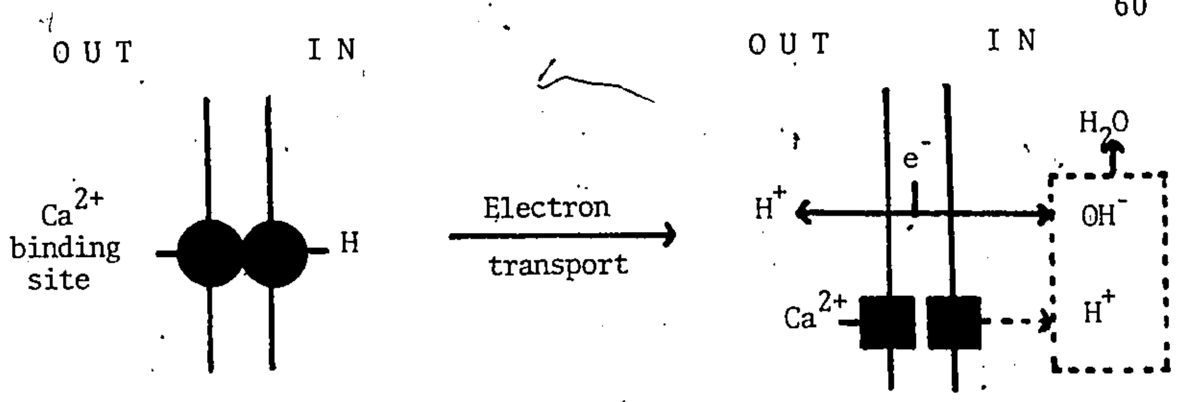

Figure 14. Postulated Mechanism of Membrane Loading of Ca^{2+} to the Inner Mitochondrial Membrane

Ca^{2+} binding sites, which are located on specific proteins in the inner membrane, are suggested to contain protonated groups exposed to the inner or matrix side of the inner membrane. In the absence of electron transport, these groups remain protonated and in this state the binding sites have a low-affinity for Ca^{2+}. Electron transport converts the low-affinity sites to high-affinity sites by deprotonating the Ca^{2+} binding proteins on the inside.

(From Lehninger, 1972)

collapse the membrane gradient, causing the Ca^{2+} binding proteins to revert to a low-affinity form.

The membrane loading of calcium consists of two phases (Lehninger, 1974). When respiring mitochondria are exposed to Ca^{2+} there is a very fast respiration-dependent binding of Ca^{2+} accompanied by H^+ ejection, which occurs prior to the onset of stimulated oxygen consumption. This rapid phase of Ca^{2+} binding is essentially complete before Ca^{2+} stimulated respiration occurs. It is followed by a slower phase of Ca^{2+} accumulation that is proportional to and thus presumably stoichiometric with the stimulated oxygen consumption.

If a permeant anion such as phosphate is present, greater than 100 nmoles Ca^{2+}/mg protein can be taken up. This process is called matrix loading and results in the formation of calcium phosphate salts in the matrix. This usually results in mitochondrial swelling (Lehninger, 1974). Lehninger (1974) has shown that the only anions capable of supporting matrix loading of calcium are those which can yield a proton, directly or indirectly, on passing into the mitochondrial matrix. These include anions such as phosphate, acetate, bicarbonate, arsenate and butyrate. As Fig. 15 shows, Ca^{2+} enters the matrix without a countercurrent of any other ion. The uptake of Ca^{2+} by the mitochondrion is powered by the membrane potential and is independent of the pH gradient. In contrast to this, the uptake of phosphate is driven only by the difference in proton concentration or Δ pH. In this case, the membrane potential

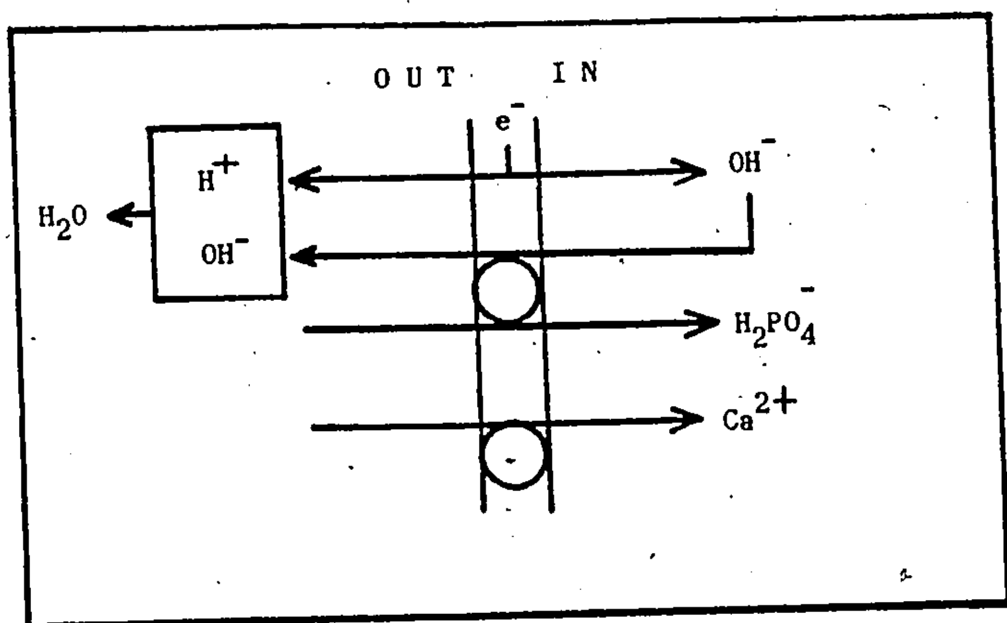

Figure 15. Postulated Mechanism of Calcium Uptake in the Presence of Phosphate into the Mitochondrial Matrix

Respiration produces a Δ pH which is alkaline inside. Phosphate and certain other ions may be pulled electrophoretically into the alkaline matrix of respiring mitochondria because of their capacity to donate protons. As shown, $H_2PO_4^-$ may exchange for matrix hydroxyl ions on the phosphate-hydroxide antiporter. This reaction is equivalent to the entry of undissociated H_3PO_4, followed by loss of a proton to the alkaline matrix. Moreover, once the $H_2PO_4^-$ ion has gained entry into the matrix it may on further dissociation yield additional protons to the alkaline matrix and thus further aid in generating a phosphate gradient. The net effect of the entry of phosphate into the matrix is to convert the alkaline-inside electrochemical gradient of protons generated by electron transport into a negative-inside gradient. The internal excess of phosphate generated in this way is the immediate "pulling" force for electrophoretic transport of Ca^{2+} from the medium into the matrix on the specific electrogenic Ca^{2+} uniporter.

(From Lehninger, 1972)

has no effect, since while it accelerates the outward passage of hydroxyl ions it retards the inward movement of phosphate and the two effects exactly cancel.

In other words, the difference in proton concentration drives the inward passage of phosphate which causes the formation of a transmembrane potential that is negative inside. This membrane potential becomes the "pulling" force for electrogenic transport of Ca^{2+} via the carrier into the matrix.

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 nmoles Ca^{2+}/mg mitochondrial protein may be taken up (Vasington & Murphy, 1962). In this case the accumulated calcium appears in the matrix as insoluble calcium phosphate granules (Lehninger, 1970). The presence of ADP or ATP, in addition to a respiratory substrate, is required to retain the accumulated calcium (Kimura & Rasmussen, 1977; Sordahl & Asimakis, 1978). If ADP is omitted, the mitochondria simply swell and fail to accumulate more than a few hundred nmoles of Ca^{2+}/mg protein. Therefore, when calcium is accumulated, there is a simultaneous accumulation of adenine nucleotides from the medium and these nucleotides play a key role in the retention of the accumulated calcium by the mitochondria. Carafoli et al. (1965) found that the amounts of adenine nucleotides taken up from the suspending medium may approach 8 to 9 times the normal level in mitochondria.

Accumulation of Ca^{2+} under massive loading conditions causes irreversible damage to oxidative phosphorylation and respiratory control mechanisms as well as characteristic morphological changes (Greenawalt et al., 1964).

Another important characteristic of mitochondrial calcium uptake is that it usually takes precedence over ADP phosphorylation (Lehninger, 1970). If as little as 10 μM Ca^{2+} is added to mitochondria in the presence of 3 mM ADP, concentrations which are in the physiological range for each, the mitochondria will very rapidly and exclusively accumulate Ca^{2+} until it is almost totally removed from the medium before begining the phosphorylation of the added ADP. Primacy of Ca^{2+} transport over oxidative phosphorylation has been found in all vertebrate mitochondria studied to date except heart mitochondria. In these mitochondria calcium upake and ADP phosphorylation occur with equal affinities (Jacobus et al., 1975).

There has been considerable interest in determining what natural occuring compounds are capable of releasing Ca^{2+} once it has been accumulated. In 1974 Borle reported that cyclic AMP in low concentrations could induce a rapid and complete release of calcium from previously-loaded liver and kidney mitochondria (Borle, 1974). However, his results could not be reproduced by a number of laboratories (Scarpa et al., 1976) and later by Borle himself (Borle, 1976). Therefore, the initial results obtained by Borle in 1974 appear to be in error. In contrast to the lack of effect of cyclic AMP on mitochondrial

calcium release, sodium ion was shown to be capable of releasing calcium from heart mitochondria (Carafoli et al., 1974; Crompton et al., 1978). Crompton et al. (1978) have reported the presence of a carrier-mediated exchange between Ca^{2+} and Na^{+} in mitochondria of skeletal muscle and some secretory tissues (Fig. 16). When the energy-linked uptake process for Ca^{2+} is completely inhibited by ruthenium red, Na^{+} induces a rapid efflux of the Ca^{2+} accumulated by mitochondria isolated from a number of tissues. The mitochondria that exhibit Na^{+}-induced efflux of Ca^{2+} also contain Na^{+}/H^{+} exchange activity.

(2) Magnesium Transport

Mitochondria isolated from heart are able to accumulate large amounts of Mg^{2+} (Brierley et al. 1962, 1963). The respiration-dependent process occurs most rapidly in the presence of phosphate which results in the formation of $Mg_3(PO_4)_2$ in the matrix, although some Mg^{2+} can be taken up in the absence of phosphate. The accumulation of Mg^{2+} is abolished by inhibitors of respiration, by dinitrophenol and other uncouplers, but not by oligomycin.

The energy-dependent accumulation of Mg^{2+} by heart mitochondria has quite different characteristics from the uptake of Ca^{2+} (Crompton et al. 1976). Mg^{2+} does not normally stimulate oxygen consumption by resting mitochondria and is not accumulated under limited-loading conditions favourable for uptake of Ca^{2+}. Accumulation of Mg^{2+} is abolished by addition

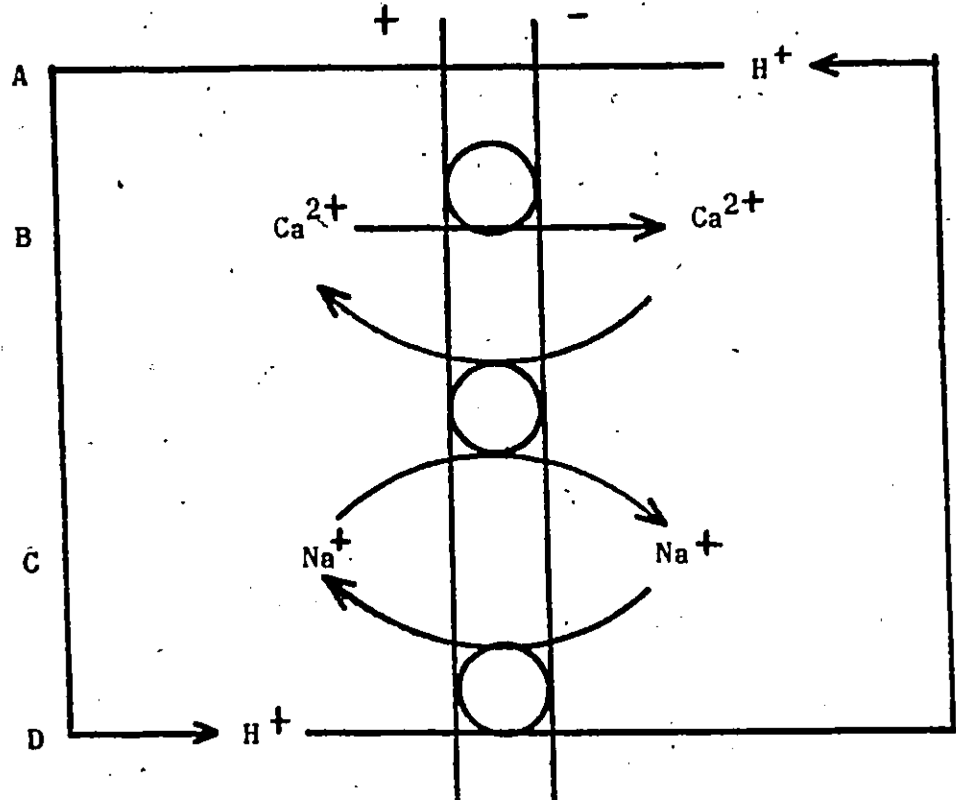

Figure 16. The Interrelations Between the Fluxes of Na^+, H^+ and Ca^{2+} in Mitochondria Isolated from Various Tissues

(A) Respiration-dependent efflux of H^+; (B) the ruthenium red-sensitive influx of Ca^{2+}; (C,D) Na^+-Ca^{2+} and Na^+-H^+ antiporters respectively. Addition of ruthenium red to mitochondria isolated from brain, adrenal cortex, parotid gland and skeletal muscle inhibits the further uptake of Ca^{2+} by these mitochondria but induces little or no net Ca^{2+} efflux. The further addition of Na^+, however, induces rapid efflux of Ca^{2+}.

(From Crompton et al., 1978)

of Ca^{2+} or ADP (Lehninger, 1967) and the uptake of Mg^{2+} heart mitochondria is not supported by ATP and is not inhibited by ruthenium red.

These results suggest that the mechanisms of the uptake of Mg^{2+} and Ca^{2+} may be distinct although it cannot be concluded that they are completely independent processes. Jacobus et al. (1975) have shown that Mg^{2+} ions do bind to the transport system for Ca^{2+} in heart mitochondria. However, Crompton et al. (1976) have shown that the affinity for Mg^{2+} of the transport system for Ca^{2+} is very low. Schuster and Olson (1974) stated that magnesium transport across mitochondrial membranes consists of at least three phases:

a) an energy-independent binding of the divalent metal to the surface of the membrane

b) an energy-dependent movement of the metal ion across the membrane

c) an anion-dependent transfer of the metal ion from the membrane into the matrix space.

It is possible that Mg^{2+} uptake might not be carrier-mediated but there is little firm basis to reach a definitive conclusion at this time (Brierley, 1976).

Crompton et al. (1976) reported that energy-linked respiration can elicit an efflux of Mg^{2+} from heart mitochondria by a process that may require phosphate. When the extramitochondrial concentration of Mg^{2+} was less than 2.5 mM an efflux of Mg^{2+} was observed, whereas a net influx of Mg^{2+}.

occurred when the external concentration of Mg^{2+} was greater than this.

Rat liver mitochondria accumulate Mg^{2+} and phosphate rather poorly under conditions which result in massive accumulation in heart mitochondria (Lehninger, 1967). In contrast to the accumulation of Mg^{2+} by heart mitochondria, Mg^{2+} uptake by liver mitochondria is an ATP-dependent process and is inhibited by ruthenium red. Oligomycin at concentrations sufficient to inhibit phosphorylation coupled electron transport and ATP synthesis does not modify Mg^{2+} flux, which is dependent on added ATP. Kun (1976) postulated the existence of an oligomycin insensitive ATPase, serving as a Mg^{2+} pump.

4. Ultrastructural Changes in Mitochondria after Calcium Uptake

Hackenbrock and Caplan (1969) used electron microscopy to observe the conformational changes in mitochondria caused by calcium uptake. They noted that calcium uptake in the absence of phosphate did not cause any appreciable increase in the volume of the inner mitochondrial compartment. However, when phosphate was present the addition of calcium caused the inner mitochondrial compartment to expand in proportion to the quantity of ion accumulated.

Hunter et al. (1976) concluded that the increase in volume of the inner mitochondrial compartment was due to an increase in the permeability of the membrane caused by the displacement of Mg^{2+} at specific sites by Ca^{2+} ions. They noted that the increase in permeability of the inner membrane was paralleled

by an increased uncoupling in calcium-treated mitochondria and conditions which restored coupling also restored the impermeability of the membrane.

Light scattering methods have been used in several laboratories to ascertain that the energized accumulation of Ca^{2+} by mitochondria leads to mitochondrial swelling (Chappell & Crofts, 1965; Azzi & Azzone, 1966; Siliprandi et al,, 1975). Decrease in the light-scattering properties of mitochondria under prescribed conditions of Ca^{2+} accumulation is consistent with the idea that ion movements can initiate water movements in mitochondria (Lehninger, 1967). However, light scattering methods have been criticized by Hackenbrock and Caplan (1969) who state that the nonspecificity of light scattering leaves doubt as to what per cent of the mitochondria of a suspension "swells" and which compartment in the mitochondria undergoes "swelling" during ion accumulation.

A number of investigators have suggested that a large part of the decrease in optical density associated with energy-linked swelling of mitochondria occurs as a result of osmotically-induced expansion of the inner membrane-matrix compartment within the intact outer membrane (Hackenbrock, 1968; Massari et al., 1972). In an attempt to resolve the problem of which compartment actually swells during the decrease in absorbance Schmidt et al. (1977) used the Coulter Counter to measure directly the changes in overall mitochondrial volume during energy-linked swelling. The Coulter Counter, which counts and sizes small suspended particles, has been used by several authors

to measure mitochondrial volume (Gear & Bednarek, 1972; Glas & Bahr, 1966; Gebicki & Hunter, 1964).

Schmidt et al. (1977) determined that although 84% of the total swelling, as measured by optical density measurements, was complete within 3 minutes, overall mitochondrial volume, as determined with the Coulter Counter, increased only 20%. They concluded that energy-linked swelling proceeds in three distinct phases (Fig. 17):

1. Phase I is the time of onset and is characterized by the condensed conformation of the mitochondria and both volume and optical density changes are minimal.

2. Phase II is the rapid swelling phase which is characterized by a large decrease in optical density but with only minor changes in overall volume. During this phase there are dramatic alterations in the inner membrane-matrix space as reflected by the transition from the condensed to twisted to orthodox conformation. This phase is energy-linked.

3. Phase III is termed the slow-swelling phase and is the time during which actual mitochondrial swelling takes place. Volume increases are rapid during the initial period of this stage, whereas optical density changes are minimal.

These results indicate that optical density changes during swelling are a reflection of expansion of the inner membrane-matrix compartment within the intact outer membrane. However, there is one criticism to be made concerning this study. The optical density measurements were made with a mitochondrial

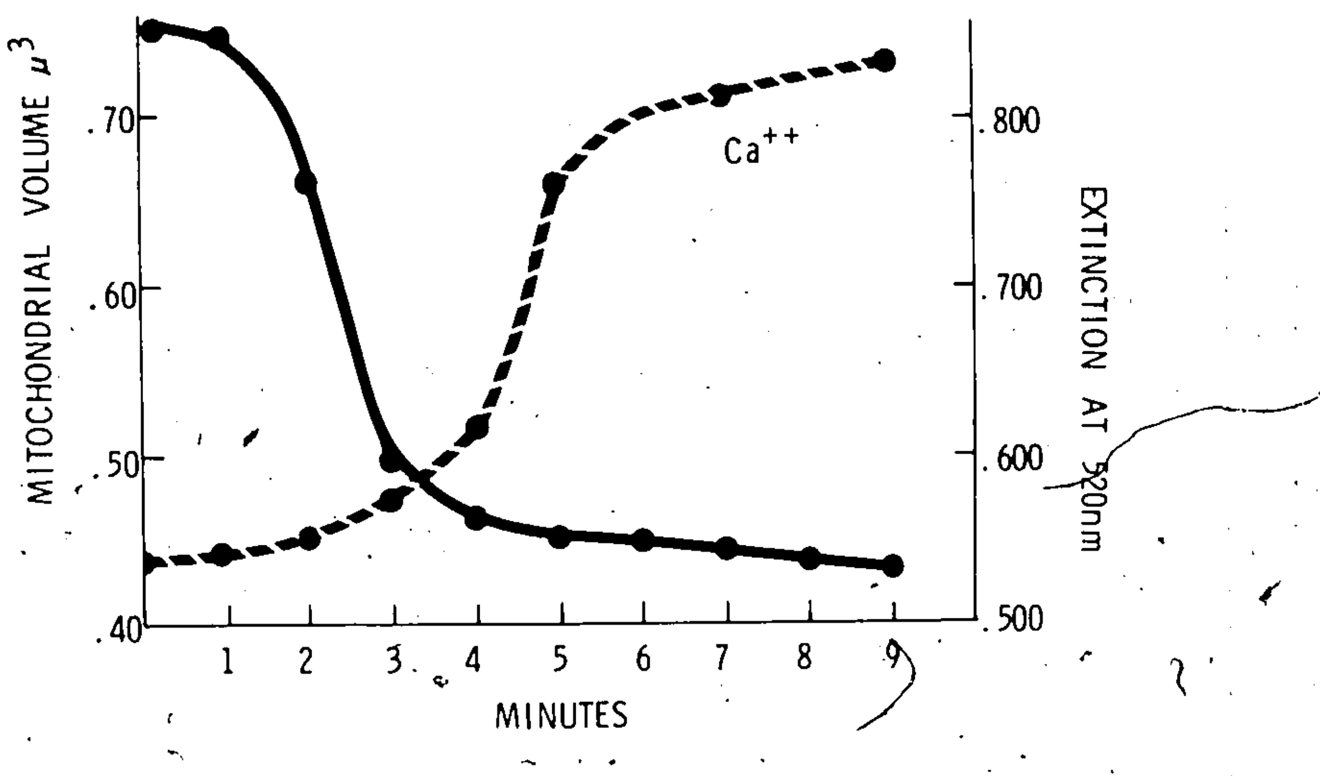

Figure 17. Time Course of Volume and Optical Density Changes of Mitochondria Suspended in $CaCl_2$

The figure compares the time course of volume changes (●---●) as measured by the Coulter Counter with the time course of optical density changes (●——●) determined at 520 nm of mitochondria suspended in 10 mM $CaCl_2$. The time course is divided into three phases: a distinct time of onset; a rapid decrease in optical density with little change detected in mitochondrial volume; and a subsequent slow decrease in optical density with a rapid change in mitochondrial volume.

(From Schmidt et al., 1977)

protein concentration of 0.2 mg protein/ml while the Coulter Counter measurements were made with a protein concentration of 0.2 μg protein/ml. It is possible that the use of two different protein concentrations could have affected the results.

5. Regulation of Monovalent Cation Transport in Mitochondria by Magnesium Ions

The electrochemical gradient generated by electron transport usually provides the driving force for cation transport. Before discussing the evidence indicating that ion transport could play a thermogenic role in brown adipose tissue mitochondria it is necessary to review the factors which are capable of regulating these ion movements.

There is ample evidence indicating that Mg^{2+} ions can control the permeability of mitochondrial membranes to monovalent cations (Chavez et al., 1977; Settlemire et al., 1968; Brierley, 1976; Binet & Volfin, 1974). Both the operation of the K^+/H^+ exchanger and the inward K^+ uniport are known to be inhibited by Mg^{2+} (Chavez et al., 1977). It is thought that specific pathways for electrophoretic penetration of monovalent cations are present in the inner membrane of the mitochondrion and that Mg^{2+} bound by a limited number of high-affinity sites in or near these pathways can control monovalent cation permeability. The experiments of Wehrle et al. (1976) support this hypothesis. They showed that removal of Mg^{2+} by addition of high levels of EDTA altered the monovalent cation selectivity of the respiration-dependent ion accumulation reaction from

$K^+ > Na^+$ to $Na^+ > K^+$, This would then indicate that EDTA, by removing Mg^{2+}, activates recycling of Na^+ (Fig. 18). Their results are therefore compatible with the idea that specific ionophores for monovalent cation transport in mitochondria are unmasked by removal of a small amount of Mg^{2+}.

These results raise the question of how these specific pathways can be unmasked in vivo. If the presence or absence of Mg^{2+} is postulated to be a controlling factor in determining the permeability of mitochondria to other cations it is necessary to consider how the level of Mg^{2+} in mitochondria is regulated.

6. Control of Magnesium Levels in Mitochondria

Several authors have demonstrated that calcium uptake is followed by an efflux of Mg^{2+} (Hunter et al., 1976; Binet & Volfin, 1974; Siliprandi et al., 1973; Siliprandi et al., 1975). For example Binet and Volfin (1974), using rat liver mitochondria, showed that in the absence of Ca^{2+}, a limited amount of Mg^{2+} was ejected from the mitochondria (Fig. 19). However, when Ca^{2+} was added they observed a fast and total efflux of the mitochondrial Mg^{2+}. Also, at the same time the Ca^{2+}, which was initially incorporated in the mitochondria, was rapidly released into the medium (Fig. 19).

It is generally agreed that some sites in the membrane, which are initially occupied by Mg^{2+}, can be removed or displaced by Ca^{2+}. These sites are thought to be present on the external side of the inner membrane (Binet & Volfin, 1975). When

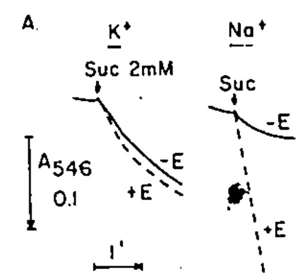

Figure 18. Effect of EDTA on Energy-Dependent K^+ or Na^+ Uptake

Osmotic swelling at 25°C was recorded at 546 nm using an Eppendorf photometer. Beef heart mitochondria (0.5 mg/ml) were treated with rotenone and added to a medium of K^+ or Na^+ acetate (20 mM), sucrose (100 mM), Tris acetate (2 mM, pH 7.0), and EGTA (30 mM), in the presence and absence of 2 mM EDTA (E). The response to the addition of Tris succinate (2 mM) is shown.

(From Wehrle et al., 1976)

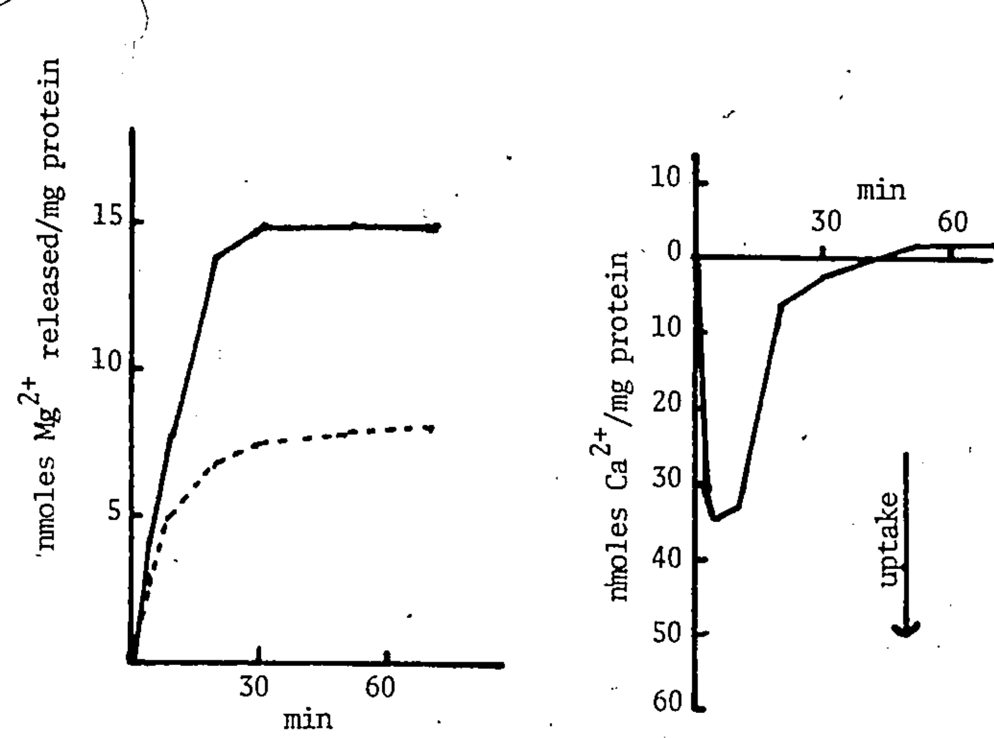

Figure 19. Kinetics of Mg^{2+} and Ca^{2+} Movements in Rat Liver Mitochondria

Mitochondria (1.8 mg protein/ml) were incubated in 0.15 M KCl, 10 mM Tris-chloride, pH 7.2, 30 μM ADP, in shaking flasks at 30°C. Reaction was started by addition of mitochondria. Aliquots were taken out, centrifuged, and Mg^{2+} and Ca^{2+} were measured by atomic absorption spectrophotometry. (●---●---●): Control (no Ca^{2+} added); (●——●——●): +100 μM Ca.

(From Binet & Volfin, 1974)

this Mg^{2+}, which is present on a number of membrane sites, is displaced by Ca^{2+}, severe modifications of certain mitochondrial functions occur such as uncoupling of oxidative phosphorylation and loss of the impermeability of the membrane to monovalent cations and other ions or molecules. If a significant amount of this Mg^{2+} is displaced by calcium, then the resulting permeability changes can cause the release of the accumulated calcium (Binet & Volfin, 1974; Binet & Volfin, 1975).

Phosphate is also known to cause an efflux of Mg^{2+} from mitochondria (Hunter et al., 1976; Crompton et al., 1976). For example, Hoser et al. (1976) demonstrated that inorganic phosphate induced a respiration-dependent delivery, from rat liver mitochondria, of mitochondrial Mg^{2+} into the medium. It was also concluded that endogenous calcium, which leaks out of the mitochondria during storage and incubation, is also required since the specific calcium transport inhibitors EDTA, ruthenium red and lanthanum prevented the release of Mg^{2+}. Since these compounds prevent re-uptake of Ca^{2+} by mitochondria it suggests that calcium recycling at the mitochondrial level is involved.

That phosphate plays an essential role in the stimulation of Mg^{2+} ejection was indicated by the inhibitory action of specific inhibitors of phosphate transport such as N-ethylmaleimide which inhibits the phosphate-hydroxyl exchange system and nigericin which prevents phosphate uptake by dissipating the transmembranal pH due to its action as a H^+/K^+ antiporter (Höser et al., 1976).

As well as being good respiratory substrates, long chain fatty acids are known to affect many mitochondrial functions (Wojtczak et al., 1969). Small amounts of oleate, about 50 nmoles/mg, inhibit the transport of ATP into the mitochondrion and cause an unspecific swelling of the mitochondrion and the release of some Mg^{2+} (Wojtczak et al., 1969), and some K^+ (Wojtczak, 1974). The swelling effect of fatty acids appears to be due to increased cation permeability and this effect is abolished by Mg^{2+} (Wojtczak, 1974). It is thought that low amounts of fatty acids induce the release of Mg^{2+} and as a consequence increase the permeability of the mitochondrion to cations as indicated by swelling and loss of K^+. Since the ionophores valinomycin and gramicidin are known to exert such an effect, an ionophoretic effect for fatty acids has been suggested by Wojtczak (1974). As with the ionophores, two mechanisms of action were described. A carrier mechanism was proposed for free fatty acids. This mechanism assumes that the penetrating species are undissociated salts of fatty acids with monovalent metals or cation fatty acid complexes. On the other hand, a channel mechanism appeared more likely for palmitoyl-CoA since it is well known that acyl CoA cannot pass the inner mitochondrial membrane.

Thus, fatty acids appear to affect the mitochondrion by some physical rather than chemical interaction with the molecular arrangement of biological membranes.

PART C: ROLE OF IONS IN ACCLIMATION TO COLD

1. Ion Transport in Brown Adipose Tissue Mitochondria

As we have seen in the last section, when the cycling of certain ions is promoted respiration is stimulated and thus phosphorylation becomes loosely coupled to respiration. This is relevant to the study of nonshivering thermogenesis since both increased cycling of calcium (Hittelman et al., 1967; Christiansen, 1971) and increased cycling of potassium (Fain & Reed, 1970) have been proposed as thermogenic mechanisms in brown adipose tissue mitochondria.

The evidence so far available suggest that active uptake of ions in brown adipose tissue mitochondria requires respiration in the presence of factors which promote tight coupling. Hittelman et al. (1967) showed that mitochondria isolated from brown adipose tissue of cold-acclimated rats are capable of accumulating Ca^{2+} in the presence of ATP or ADP and substrate by means of respiration-dependent mechanism. However, since Ca^{2+} uptake in the presence of substrate and ATP was found to be inhibited by oligomycin and since it was shown in a previous experiment (Smith et al., 1966) that oligomycin inhibited ATP hydrolysis in these mitochondria, Hittelman et al. (1967) concluded that ADP was the active form of the nucleotide in maintaining Ca^{2+} uptake. Christiansen (1971) studied the release of controlled respiration with low concentrations of calcium in brown adipose tissue obtained from guinea pigs. He showed that in the absence of a nucleotide, mitochondria from

non-thermogenic brown adipose tissue (fetal & weaned animals) displayed a normal respiratory response to added calcium while mitochondria from thermogenic brown adipose tissue (newborn & cold-acclimated) hardly responded. However, the addition of a nucleotide di- or triphosphate converted the mitochondria of thermogenic brown adipose tissue to a type capable of respiratory release with added clacium. He concluded that the calcium of brown adipose tissue was metabolically active and that it could play a role in the respiratory events which lead to thermogenesis in brown adipose tissue.

Christiansen (1971) also demonstrated that brown adipose tissue mitochondria are unique in their low capacity for utilizing ATP for energizing calcium uptake in the absence of respiration and hence supports the conclusion that these mitochondria have a low capacity for oxidative phosphorylation.

Thus, brown adipose tissue mitochondria are capable of actively transporting calcium. If accumulated calcium were allowed to leave the matrix it would then be available for active re-uptake. Such a futile cycle could represent a major thermogenic process. The fact that brown adipose tissue mitochondria possess a Na^+ -dependent efflux pathway for calcium (Nicholls, 1978) indicates that a high rate of calcium recycling is possible.

Fain and Reed (1970) proposed that increased cycling of potassium could be a potentially significant thermogenic mechanism. As was the case with calcium transport, the active transport of potassium by brown adipose tissue mitochondria

also requires respiration in the presence of factors which promote tight coupling (Thomassen et al., 1975). In contrast to the active transport of calcium and of potassium in mitochondria isolated from a variety of tissues, the active transport of potassium in brown adipose tissue mitochondria is not accompanied by any detectable volume changes. This result led Thomassen et al. (1975) to conclude that the energy-dependent uptake of potassium is followed by the release of potassium due to the permeability of these mitochondria to this ion. In this case, the electrical potential gradient established through respiration would be directly counterbalanced by the potassium influx which would leave the proton gradient intact.

The available evidence therefore indicates that brown adipose tissue mitochondria are capable of actively transporting calcium and potassium ions and that increased cycling of either ion could provide a thermogenic mechanism.

Since Section 5 pointed out that magnesium ions could regulate ion transport it would be useful to review what is known about the effect of cold-acclimation on the ionic content of mitochondria isolated from brown adipose tissue and other tissues.

2. Ionic Changes in Mitochondria as a Result of Cold-Acclimation

Most of the ionic content measurements of mitochondria isolated from cold-acclimated rats have been made using liver mitochondria. These studies were initiated at the time when it was believed that liver was a site of NST. Smith and

Fairhurst (1958) determined that cold-acclimation resulted in an enhancement of liver mitochondrial state 3 respiration (respiration in the presence of added ADP). Although the mechanism by which this enhancement occurs has not been fully determined it is known that ions can influence mitochondrial metabolism (Bygrave, 1967) and that alterations in the content of intracellular ions could play a central role in the control of cellular respiration. Bramante and Nirdlinger (1974) have measured the Na^+, K^+, Ca^{2+} and Mg^{2+} levels of liver mitochondria isolated from warm- and cold-acclimated rats. There was a small but significant difference found in the calcium content which indicated that cold-acclimation results in a decreased amount of this ion. Although the meaning of this difference is not readily apparent these results did not support the hypothesis that massive calcium accumulation is the major mechanism operative in the maintanance of NST in liver mitochondria. Bramante and Nirdlinger (1974) suggested that the lower calcium concentrations found in liver mitochondria isolated from cold-acclimated rats could be an indication of a decreased mitochondrial calcium sequestering ability, which was caused by uncoupling of oxidative phosphorylation. McBurney and Radomski (1973) measured the calcium and magnesium levels in liver mitochondria isolated from warm- and cold-acclimated rats in the presence or absence of EDTA. When the mitochondria were isolated without EDTA they found that the level of magnesium was higher in the warm- than in the cold-acclimated

preparations with little difference found in the calcium levels. The addition of 1 mM EDTA to the homogenization solution reduced the level of magnesium and calcium to a greater extent in liver mitochondria isolated from cold-acclimated rats than liver mitochondria isolated from warm-acclimated rats (Table V11). McBurney and Radomski (1973) suggested that a possible explanation for these findings was that liver mitochondria obtained from cold-acclimated rats possess larger labile pools of calcium and magnesium than mitochondria obtained from warm-acclimated rats, due to an uncoupling of liver mitochondria as a result of cold-acclimation.

There have been only two reports on the ionic change in mitochondria from BAT as a result of cold-acclimation. Both studies showed that cold-acclimation results in a decreased amount of mitochondrial calcium. Greenway and Himms-Hagen (1978) found that IBAT mitochondria isolated from warm- and cold-acclimated rats contained 44 and 20 nmoles/mg mitochondrial protein respectively. There results were in agreement with those of Christiansen (1971) who found that BAT mitochondria isolated from weaned (non-thermogenic) and cold-acclimated (thermogenic) guinea pigs contained 81.1 and 12.2 nmoles/mg mitochondrial protein respectively.

The results obtained using liver and BAT mitochondria suggest that cold-acclimation results in an increase in the permeability of the inner mitochondrial membrane. Because of the involvement of magnesium ions on the permeability of mito-

TABLE VII

Calcium and Magnesium Contents of Liver Mitochondria Prepared in the Absence and Presence of EDTA

Cation	1.0 mM EDTA	WA (nmoles/mg protein)	CA
Calcium	-	5.47 ± 0.16 (15)	5.59 ± 0.20 (17)
	+	3.35 ± 0.24 (10)	2.43 ± 0.12 (9)
		2.12 (39%)	3.16 (56%)
Magnesium	-	42.69 ± 0.53	41.63 ± 0.53
	+	29.51 ± 0.66	23.85 ± 1.18
		13.17 (31%)	17.79 (43%)

The table shows the calcium and magnesium contents of liver mitochondria obtained from warm- and cold-acclimated rats. Mitochondria were prepared in one of two homogenizing solutions: 0.25 M sucrose, 25 mM Tris-HCl, 1.0 mM EDTA, pH 7.2; or 0.23 M mannitol, 0.07 M sucrose, 25 mM Tris-HCl, pH 7.2. Results have been converted from μg/mg N to nmoles/mg protein. To convert from mg N to mg protein the former value was multiplied by 6.25. This was done because most proteins contain about 16% nitrogen so that the weight of the protein nitrogen multiplied by 6.25 (100 ÷ 16) equals the weight of the protein. Number of rats in each experimental group appears in brackets. Percentage decrement in cation in presence of EDTA appears in underlined brackets.

From McBurney and Radomski (1973)

chondrial membranes most of the experiments described in this thesis are concerned with comparing the magnesium content of mitochondria isolated from warm- and cold-acclimated rats to determine if this ion might be involved in the mechanism of NST in cold-acclimated rats.

CHAPTER III: MATERIALS AND METHODS

PART A: MATERIALS

1. Rats

Male white rats of the Holtzmann strain weighing 150-200 g were purchased from Canadian Breeding Laboratories. Upon arrival they were kept at room temperature (27° ± 1°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° ± 1°C. Rats had free access to food and water, and artifical 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, unless otherwise stated, to ensure that full acclimation to cold had been achieved. Rats were killed by decapitation.

2. Chemicals

The following chemicals were obtained from the Sigma Chemical Co.: ADP (adenosine 5'-diphosphate, sodium salt), EDTA (ethylenediaminetetracetic acid, free acid), HEPES (N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid), sucrose, bovine serum albumin (fatty acid free), succinic acid, Tris (trishydroxymethylaminomethane, acid), NTA (nitrilotriacetic acid), oxaloacetic acid, β NADH (β-diphosphopyridine nucleotide,

disodium salt), NADP (diphosphopyridine nucleotide phosphate, monosodium salt), calcium chloride, rotenone, and trichloroacetic acid.

Lanthanum chloride ($LaCl_3$: 6 H_2O), calcium and magnesium reference solutions (1000 ppm, for atomic absorption spectrophotometry), sodium hydroxide, potassium hydroxide and magnesium sulfate were obtained from Fisher Scientific Co.

Potassium phosphate (monobasic) and hydrochloric acid were obtained from Baker Analyzed Reagents.

PART B: METHODS

1. Isolation of Interscapular Brown Adipose Tissue Mitochondria

Interscapular brown adipose tissue mitochondria were isolated by the procedure of Slindo et al. (1975) except that EDTA and HEPES were omitted after the first mitochondrial washing.

Interscapular brown adipose tissue (IBAT) was excised, cleaned of white fat and skeletal muscle, weighed and chopped finely with scissors. The mince was placed in 20 ml/g of cold isolation medium and gently homogenized with a motor driven teflon-glass homogenizer (Dual #24) at 400 rpm. The isolation medium consisted of 0.25 M sucrose, 0.2 mM EDTA and 1.0 mM HEPES. The pH was brought to 7.2 with potassium hydoxide. Centrifugations were performed in the Sorvall RC-2B refrigerated centrifuge using the HB-4 rotor. The homogenate was diluted with isolation medium to 42 ml and centrifuged for 10 minutes at 4,000 x g and the resulting supernatant was filtered through 2 layers of gauze. The sediment was resuspended and homogenized

again at 400 rpm and resedimented as described above. The combined supernatants were then centrifuged for 14 minutes at 16,300 x g. The supernatant was discarded and the walls of the centrifuge tube were carefully wiped with a tissue to remove adhering fat. The mitochondrial pellets were resuspended in a small amount of isolation medium by gently stirring with a glass rod and then homogenized by hand using a teflon-glass homogenizer. After dilution with additional medium, the mitochondria were centrifuged again at 16,300 x g for 14 minutes. The pellet was resuspended in 0.25 M sucrose and recentifuged. The final pellet was resuspended in 1.0 ml of 0.25 M sucrose.

2. Isolation of Liver Mitochondria

Rat liver mitochondria were isolated by the procedure of Johnson and Lardy (1967). The isolation medium was the same as that used in isolating brown adipose tissue mitochondria.

The liver was removed and rinsed three times in isolation medium to remove excess blood. 2.5 g of liver was taken and cut into smaller pieces. After addition of 20 ml of isolation medium, the liver was homogenized with a teflon-glass homogenizer at 400 rpm. The homogenate was adjusted to 40 ml with isolation medium and centrifuged using a HB-4 rotor for 10 minutes at 650 x g and then resulting supernatant was filtered through 2 layers of gauze. The sediment was resuspended, homogenized by hand with a teflon-glass homogenizer and recentrifuged as described above. The combined supernatants were

then centrifuged for 5 minutes at 14,700 x g. The resultant supernatant was discarded along with any lightly packed pink microsomes. Lightly packed tan mitochondria were retained. The pellets were resuspended in 20 ml of isolation medium (0.25 M sucrose, 0.2 mM EDTA & 1.0 mM HEPES), and recentrifuged for 5 minutes at 14,700 x g. The pellets were washed twice with 0.25 M sucrose and finally resuspended in 1.0 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 with cold 12.5% (w/v, final concentration) trichloroacetic acid. After standing on ice for 30 minutes, the precipitated protein was centrifuged at 16,300 x g for 10 minutes and the pellet dissolved in 1.0 ml warm 0.5 N NaOH.

4. Measurement of Calcium and Magnesium in Mitochondria

The calcium and magnesium content of mitochondrial fractions was determined essentially as described by Carafoli and Lehninger (1971). To 0.5 ml of mitochondria suspended in 0.25 M sucrose was added 0.3 ml of $LaCl_3$ and HCl so that the final concentration was 1.0% (w/v) $La Cl_3$ and 0.5 N HCl. This was then placed in a boiling water bath for 10 minutes. After centrifugation (10 minutes at 16,300 x g) the supernatants

were withdrawn and the pellets extracted a second time as before. The supernatants containing the extracted calcium and magnesium were combined and the volume made up to 2.0 ml with additional 0.5 N HCl and 1.0% $LaCl_3$. The calcium and magnesium concentrations were determined using a Jarrell-Ash Model 740 Dial-Atom II atomic absorption spectrophotometer. All samples and standards contained $LaCl_3$ to suppress interference by phosphates (Willis, 1961), and all had identical acid concentrations of 0.5 N HCl.

Calcium and magnesium standards were prepared from commercial stock solutions (1000 ppm, Fisher Scientific Co.).

5. Measurement of Calcium-Induced Swelling in Mitochondria

The incubation medium used for the measurement of calcium-induced swelling in IBAT mitochondria was essentially the same as that used by Skaane et al. (1972) except that 155 mM sucrose and a nitrilotriacetic acid buffer were used, 1.0 mM $MgCl_2$ was omitted and the reaction volume was 2.0 ml. The incubation medium of Skaane et al. (1972) consisted of 105 mM sucrose, 50 mM glucose, 40 mM HEPES, 2% bovine serum albumin, 1.0 mM $MgCl_2$, 2.5 mM KH_2PO_4, 10 mM succinate, 3 M rotenone, 1.0 mM ADP and 0.35 mg mitochondrial protein in a total volume of 1.0 ml.

After addition of mitochondria (final concentration 0.2 mg ml) to the incubation medium a 3.0 minute warm up (from 0°C to 25°C) was allowed before the addition of calcium. The decrease in absorbance was followed at 540 nm and 25°C with a Gilford

spectrophotometer #2400-2.

Because it was of interest to compare the response of liver mitochondria to added calcium with the response of IBAT mitochondria to added calcium the same method was used for measuring calcium-induced swelling in liver mitochondria as was used for measuring calcium-induced swelling in IBAT mitochondria.

Among the various problems associated with any _in vitro_ study involving calcium transport there are two which are relevant to this experiment (Reed & Bygrave, 1975). The first is that it is impossible to ensure that incubation systems are completely free of endogenous calcium, which causes significant dilution errors at low concentrations of calcium and secondly, calcium is weakly chelated by a large variety of organic materials including dicarboxylic acids (such as the added respiratory substrates), ATP and phosphate. However, both of these problems may be solved by using a "calcium buffer" system which ensures accurately known concentrations of calcium in the micromolar range. Since the apparent K_m for the calcium carrier system is within the range of 10^{-6}-10^{-5} M (Bygrave et al., 1971), a suitable buffer must generate stable concentrations of free calcium within this range. For this reason nitrilotriacetic acid (NTA) was added to the incubation medium since it has been shown (Reed & Bygrave, 1975) to "buffer" in the region of 10^{-6}-10^{-4} M free calcium.

6. Digitonin Treatment of Brown Adipose Tissue Mitochondria

The technique for disruption of the outer membranes was based on the study of Zaluska et al. (1975). To 0.5 ml of 250 mM sucrose, 1.0 mM HEPES, 0.05% serum albumin containing digitonin, was added 0.5 ml of 10 mg/ml mitochondria suspended in 250 mM sucrose and 1.0 mM HEPES to achieve a final digitonin concentration of 0.2 mg/mg mitochondrial protein. The resulting mixture was gently stirred at 0°C for 15 minutes. The mixture was then diluted by addition of 3.5 ml of the incubation medium without digitonin (250 mM sucrose, 1.0 mM HEPES and 0.05% serum albumin) and centrifuged at 10,400 x g for 10 minutes to sediment mitochondria and mitoplasts. The pellets were resuspended in incubation medium without digitonin and both the pellets and supernate were brought to a 5.0 ml volume and sonicated for 3 x 30 seconds. 3.0 ml were removed, acid extracted as described in Methods 3 and brought to a 5.0 ml volume for magnesium determination. The remaining 2.0 ml were used for the determination of enzyme activities. To obtain 100% activity, 0.5 ml of 10 mg/ml mitochondria was added to 0.5 ml of incubation medium and then brought to a volume of 5.0 ml with incubation medium without digitonin. The resulting suspension was sonicated and divided into two lots, one for acid extraction and the other for the determination of enzyme activity as described above. Adenylate kinase was used as a marker for the intermembrane space (Schnaitman & Greenawalt, 1968; Sottocasa et al., 1967). Malate

dehydrogenase was used as a marker for the mitochondrial matrix (Schnaitman et al., 1967). Adenylate kinase activity was measured according to Sottocasa et al. (1967) in the pellet fraction to determine the effectiveness of digitonin-induced rupture of the outer membrane. Malate dehydrogenase was measured according to the procedure described by the same authors as above except that 1×10^{-3}M amytal was also added (Schnaitman et al., 1967). Its activity was measured in the supernatant fraction to determine the extent of rupture of the matrix compartment. The reason why adenylate kinase activity was measured in the pellet fraction and not in the supernate fraction was due to the finding of Schnaitman & Greenawalt (1968), that adenylate kinase is quite labile at low protein concentrations.

Digitonin was recrystallized once from hot absolute ethanol, dried and ground to a fine powder. All digitonin containing solutions were prepared shortly before use.

7. Statistical Analysis of Results

Results are expressed as means ± standard error of the mean (SEM). Significance of differences in mean was measured using the unpaired t-test.

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-INDUCED SWELLING RATES IN INTERSCAPULAR BROWN ADIPOSE TISSUE MITOCHONDRIA ISOLATED FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

The purpose of this experiment was to study one aspect of calcium transport, namely, calcium-induced swelling, in mitochondria isolated from warm- and cold-acclimated rats to see if any differences existed which might indicate an increased capacity to dissipate energy and thus provide heat.

Since cold-acclimation has been shown to induce changes in the mitochondria of brown adipose tissue, allowing them to be better suited to a thermogenic function, much information can be obtained by seeking differences in properties between mitochondria which do not have a large thermogenic capacity (i.e. BAT mitochondria isolated from the WA rat) and mitochondria which possess a large thermogenic capacity. (i.e. BAT mitochondria isolated from the CA rat).

The reason for studying any property of calcium transport is that the movement of calcium across the inner membrane of the mitochondrion is an energy requiring process. Calcium uptake

could thus provide a potential energy dissipating process in mitochondria of the cold-acclimated rat and therefore be the basis of a mechanism for increasing oxygen consumption and heat production in nonshivering thermogenesis.

2. Description of the Experiment

A total of 15 rats, 12 warm-acclimated (WA) and 3 cold-acclimated (CA), had lived approximately 2 months at their respective acclimation temperatures before the start of the experiment. The mean weights of the rats were: WA: 478 ± 9g; CA: 421 ± 16g.

The reason why more WA rats were required than CA rats was the lower quantity of IBAT and the lower quantity of mitochondria per cell present in the WA rat (Flatmark & Pederson, 1976).

The homogenization of IBAT and the first mitochondrial washing were performed using 0.25 M sucrose, 0.2 mM EDTA and 1.0 mM HEPES at pH 7.2. 0.25 M sucrose was used for the remaining two mitochondrial washings to remove the EDTA which would affect the concentration of free calcium generated by the nitrilotriacetic acid (NTA) buffer (see Methods 5). The incubation medium used for measuring rates of calcium-induced swelling consisted of 155 mM sucrose, 40 mM HEPES, 2% bovine serum albumin, 2.5 mM KH_2PO_4, 10 mM succinate, 3 μM rotenone, 1 mM ADP and 10 mM NTA buffer at pH 6.8 and 25°C. After addition of 1.0 ml incubation medium (final concentration as stated above) to 1.0 ml mitochondria stored in 0.25 M sucrose

(final concentration 0.2 mg/ml) a 3.0 minute incubation period was allowed before the addition of various amounts of calcium (5.0-500 nmoles free calcium/mg mitochondrial protein). The rate for each particular calcium concentration was obtained by following the decrease in absorbance and measuring its slope.

3. Results and Discussion

Using IBAT mitochondria obtained from a cold-acclimated rat as an example, Figure 20 shows how the mitochondria responded to addition of calcium (50 nmoles free calcium/mg mitochondrial protein). After an initial lag period there was a linear decrease in absorbance lasting approximately 3 minutes, referred to as the first swelling, followed by a second and usually more rapid decrease in absorbance, referred to as the second swelling. Three types of measurements were made for each different calcium concentration (Fig. 20) (see appendix).

1. The rate of the first swelling
2. The rate of the second swelling
3. The time required after addition of calcium for the second swelling to occur

Figure 21 shows the rates of the first swelling just after addition of calcium. Increasing concentrations of calcium up to about 100 nmoles/mg protein resulted in increasing rates of swelling. A significantly higher rate of spontaneous swelling (i.e. no added calcium) and of calcium-induced swelling at calcium concentrations greater than 200 nmoles free calcium/mg mitochondrial protein were found in IBAT mitochondria obtained

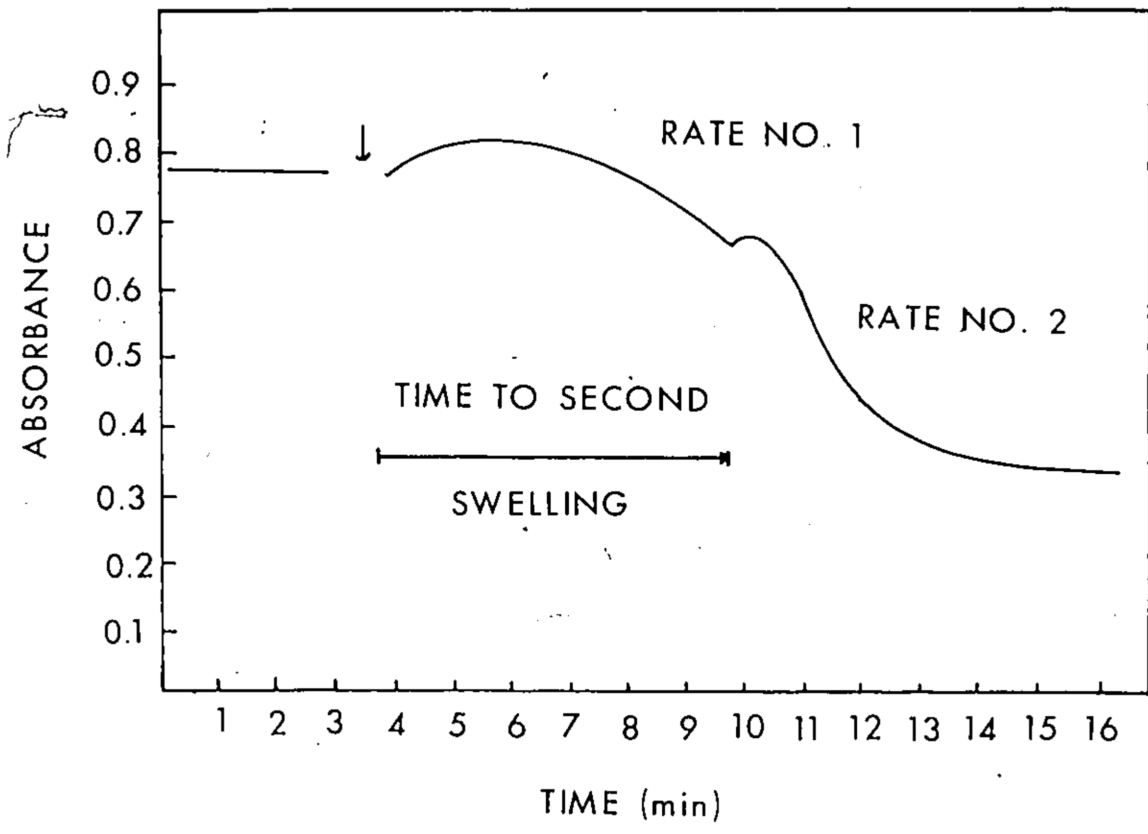

Figure 20. Decrease in Absorbance with Time of IBAT Mitochondria from Cold-Acclimated Rats in Response to Added Calcium

The figure shows the response of IBAT mitochondria isolated from cold-acclimated rats to a given amount of calcium (50 nmoles free Ca/mg protein). After addition of mitochondria to the incubation medium (155 mM sucrose, 40 mM HEPES, 2% bovine serum albumin, 2.5 mM KH_2PO_4, 10 mM succinate, 3 μM rotenone, 1 mM ADP & 10 mM NTA buffer at pH 6.8 & 25 C) a 3.0 minute incubation period was allowed before the addition of calcium (vertical arrow indicates time of addition of calcium). Rate 1 shows the initial decrease in absorbance which occurred shortly after the addition of calcium while Rate 2 represents the second period of absorbance change which occurred some time later. The time to second swelling indicates the time it took after the addition of calcium for the second period of absorbance change to occur. Changes in absorbance are interpreted as mitochondrial swelling.

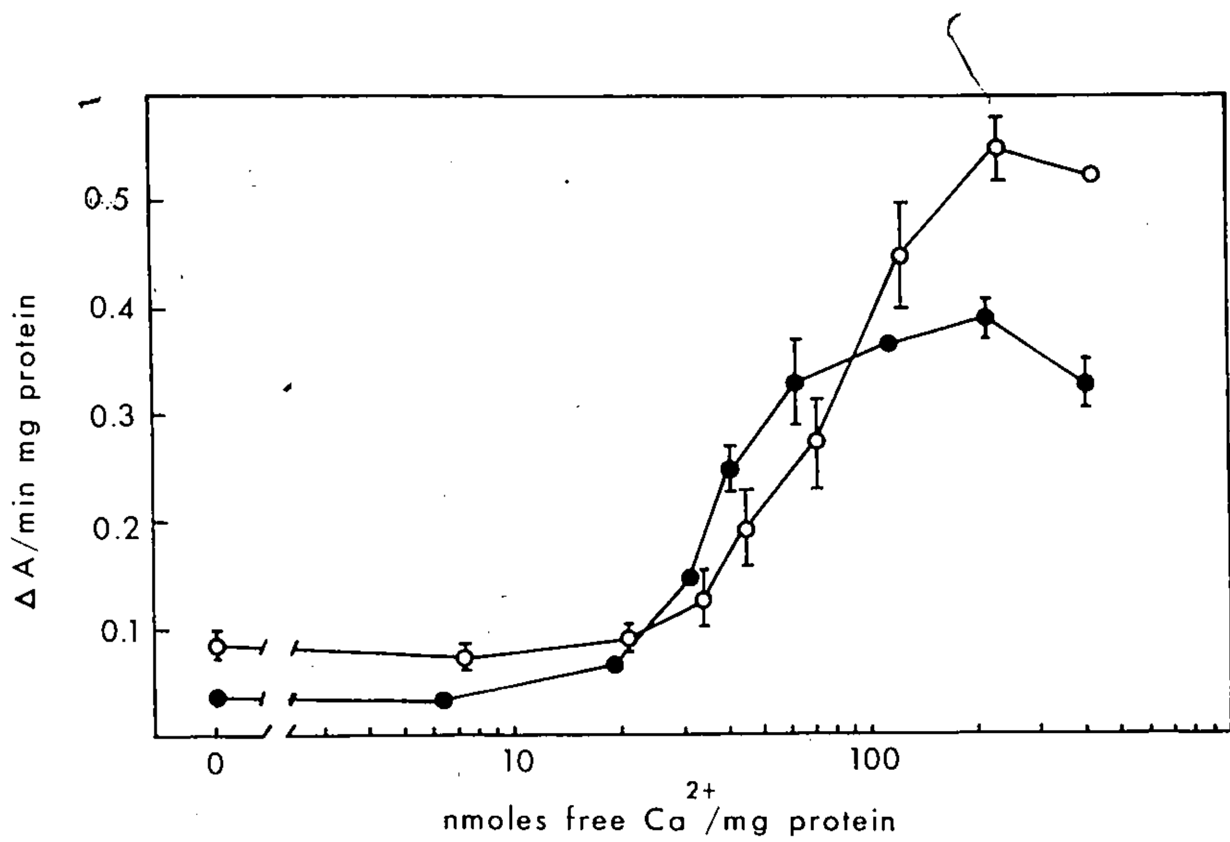

Figure 21. The Effect of Calcium Concentration on the Rate of the First Calcium-Induced Swelling in IBAT Mitochondria (Rate 1)

The figure shows rates of calcium-induced swelling as a function of free calcium concentration in IBAT mitochondria isolated from warm-acclimated [●] and cold-acclimated rats [o]. The incubation conditions were as described in Fig. 20. Free calcium was present at the indicated concentrations. Temperature was 25°C and the reaction was started by the addition of $CaCl_2$. Significant differences in the rate of calcium-induced swelling occurred at 230 nmoles free calcium/mg protein (<0.02) and at 440 nmoles free calcium/mg protein (<0.005).

from cold-acclimated rats.

Figure 22 shows the rates of the second swelling. This additional period of swelling was not expected. As shown, it occurred to a greater extent at lower calcium concentrations than the first swelling. IBAT mitochondria isolated from cold-acclimated rats displayed a significantly higher rate of second swelling compared to IBAT mitochondria isolated from warm-acclimated rats at calcium concentrations of 44 and 70 nmoles free calcium/mg mitochondrial protein.

Figure 23 shows the time between the addition of calcium and the occurrence of the second swelling. As can be seen, at a given calcium concentration the second swelling occurred sooner in IBAT mitochondria obtained from cold-acclimated rats than IBAT mitochondria obtained from warm-acclimated rats. However, this difference in time to second swelling between IBAT mitochondria isolated from warm- and cold-acclimated rats was significant only at the calcium concentration of 20 nmoles free calcium/mg mitochondrial protein.

Figure 24 shows the rates of calcium-induced swelling for the first and second swelling rates plotted on the same figure (i.e. Fig. 21 + 22). Figure 24 indicates that if 100 nmoles free calcium/mg mitochondrial protein or more was added to the incubation medium the rate of the second swelling was equal to the rate of the first swelling so that the second swelling was no longer apparent.

The finding that there is an increased rate of calcium-

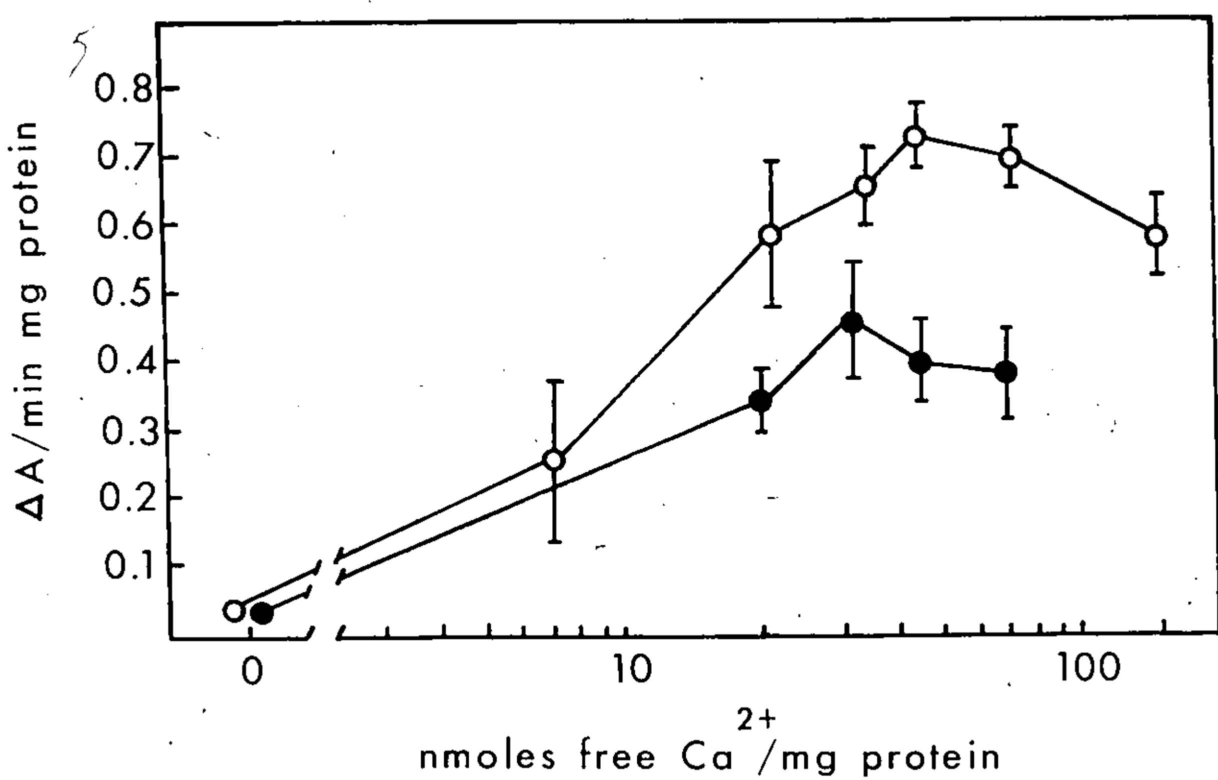

Figure 22. The Effect of Calcium Concentration on the Rate of the Second Calcium-Induced Swelling in IBAT Mitochondria (Rate 2)

The figure shows rates of calcium-induced second swelling as a function of free calcium concentration in IBAT mitochondria isolated from warm-acclimated [●] and cold-acclimated rats [○]. The incubation conditions were as described in Fig. 20. Free calcium was present at the indicated concentrations, the temperature was 25°C and the reaction was started by the addition of $CaCl_2$. Significant differences in the rate of calcium induced swelling occurred at 42 nmoles free calcium/mg protein (<0.02) and at 71 nmoles free calcium/mg protein (<0.025)

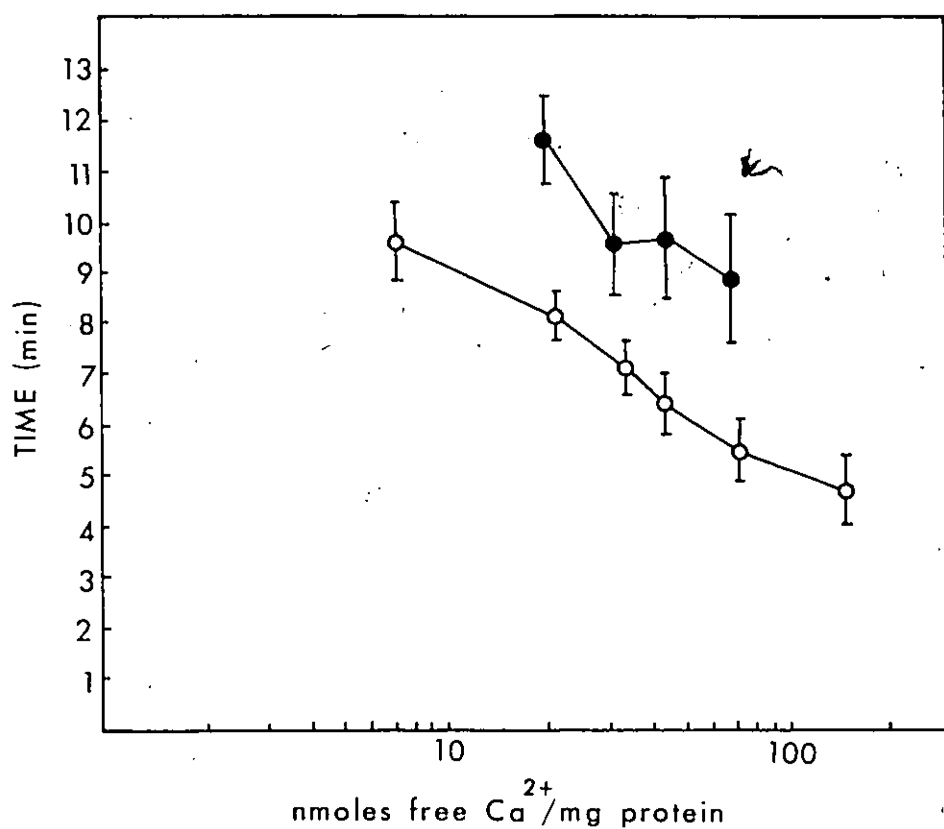

Figure 23. Time Taken After Addition of Calcium for the Second Calcium-Induced Swelling to Occur

The time taken after addition of calcium for the second calcium-induced swelling to occur in warm-acclimated [●] and cold-acclimated rats [o] is plotted against the concentration of free calcium/mg mitochondrial protein. Significant differences in the time taken after addition of calcium for the second calcium-induced swelling occurred at 20 nmoles free calcium / mg protein (<0.025).

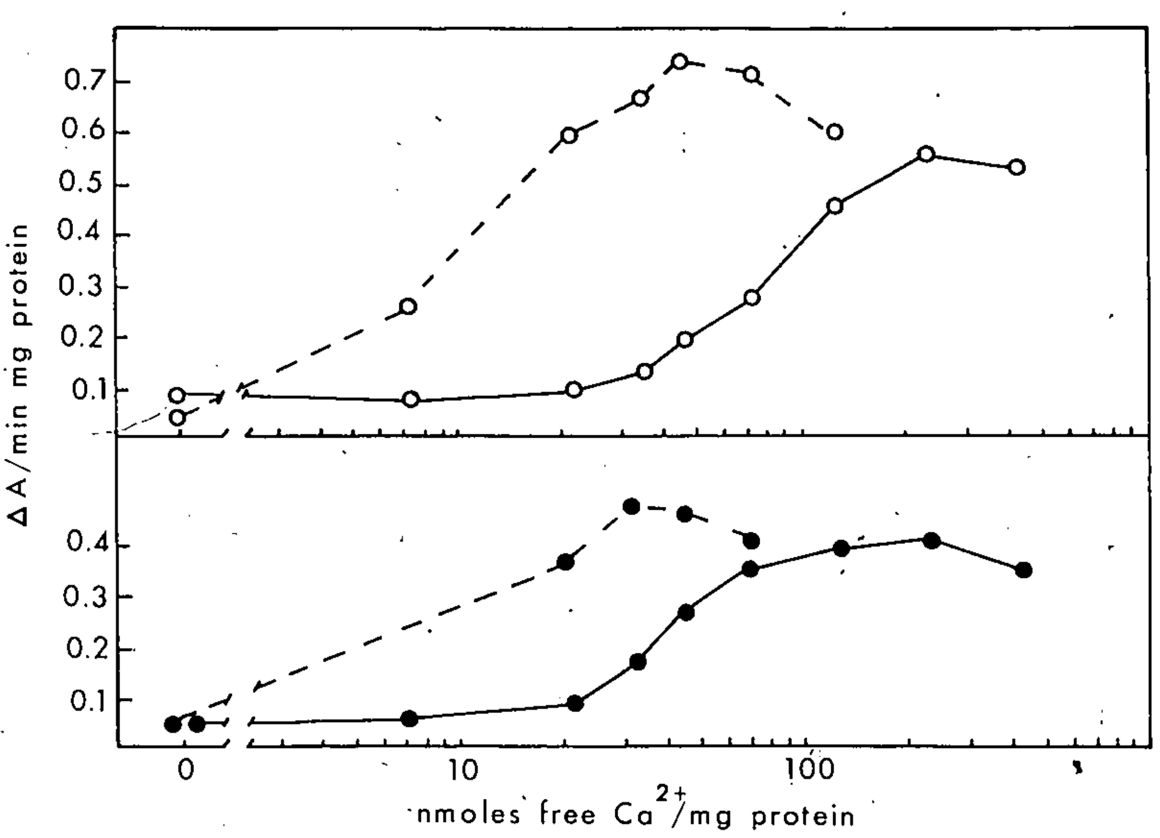

Figure 24. The Effect of Calcium Concentration on the Rate of First and Second Calcium-Induced Swelling in IBAT Mitochondria.

Figure 24 shows the rates of the first (o—o, from Fig. 21) and second (o--o, from Fig. 22) calcium-induced swellings in IBAT mitochondria isolated from cold-acclimated rats. Figure 24 shows the rates of the first (●—●, from Fig. 21) and second (●--●, from Fig. 22) calcium-induced swellings in IBAT mitochondria isolated from warm-acclimated rats.

induced swelling in IBAT mitochondria from cold-acclimated rats is of interest since it indicates that the calcium transport properties of these mitochondria are different from those of IBAT mitochondria obtained from a warm-acclimated rat. Christiansen (1971) measured the uptake of calcium in mitochondria from thermogenic brown adipose tissue of guinea pigs (newborn & cold-acclimated) and from non-thermogenic tissue (from fetal & weaned animals). He found a higher rate of uptake of calcium in mitochondria isolated from thermogenic brown adipose tissue which he postulated was the result of a more permeable membrane present in these mitochondria. Such a postulate might also account for the increased rates of calcium-induced swelling which occurred in IBAT mitochondria obtained from cold-acclimated rats.

The occurrence of a second swelling was not expected because most reports in the literature of calcium-induced swelling describe only a single phase (Azzi & Azzone, 1966; Siliprandi et al., 1975; Ramachandran & Bygrave, 1978). However, since calcium uptake is known to cause an efflux of mitochondrial magnesium (Binet & Volfin, 1974; Siliprandi et al., 1975) and since loss of mitochondrial magnesium is known to cause large volume changes as measured by light absorption (Hoser & Dargel, 1976) the possibility arose that differences in endogenous mitochondrial magnesium levels might be responsible for both the enhanced second swelling and the reduced time of its occurrence in mitochondria obtained from cold-acclimated rats. Sordahl and Asimakis (1978) and Chappell and

Crofts (1965), who used heart and liver mitochondria respectively, described a second swelling following calcium uptake which was similar to the one described here. Both studies suggested that during the second swelling all of the accumulated calcium was being released from the mitochondria. This effect could be the result of the large increase in the permeability of the mitochondrial inner membrane associated with loss of endogenous magnesium (Hunter et al., 1976; Chavez et al., 1977). The hypothesis that a difference in endogenous mitochondrial magnesium levels might be responsible for both the enhanced second swelling and the reduced time of its occurrence in mitochondria obtained from cold-acclimated rats compared to mitochondria obtained from warm-acclimated rats would imply that the magnesium content of the former would be significantly lower than that found in the latter. This possibility was explored in the next set of experiments.

PART B: MEASUREMENT OF CALCIUM AND MAGNESIUM CONTENTS OF INTERSCAPULAR BROWN ADIPOSE TISSUE MITOCHONDRIA ISOLATED FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

The previous experiment indicated that IBAT mitochondria isolated from cold-acclimated rats displayed higher rates of calcium-induced swelling than controls. It was thought that a lower level of endogenous magnesium might be responsible for this change. Therefore, the magnesium content of IBAT mitochondria was measured to see if any difference existed between

warm- and cold-acclimated rats. Calcium contents were also measured for comparison purposes.

2. Description of the Experiment

A total of 11 rats (6 wa & 5 CA) lived approximately one month at their respective temperatures before the start of the experiment. The mean weights of these rats were: WA: 412 ± 5.0; CA: 334 ± 11.0.

The procedure for this experiment is described in Methods 4.

3. Results and Discussion

The calcium content of IBAT mitochondria of cold-acclimated rats was found to be about one quarter of that of mitochondria from warm-acclimated rats (Table 8). These results are in agreement with those of Greenway and Himms-Hagen (1978) who found that IBAT mitochondria isolated from warm- and cold-acclimated rats contained 44 and 20 nmoles/mg mitochondrial protein respectively and also in agreement with the results of Christiansen (1971) who found that BAT mitochondria isolated from weaned (non-thermogenic) and cold-stressed (thermogenic) guinea pigs contained 81.1 and 12.2 nmoles/mg mitochondrial protein respectively. Christiansen postulated that the lower calcium contents found in IBAT mitochondria from cold-acclimated guinea pigs was a reflection of a more permeable membrane indicating that calcium transport may play a role in the regulatory events which lead to thermogenesis in BAT.

TABLE VIII

Calcium and Magnesium Contents of IBAT Mitochondria

IBAT (n=5)	WA	CA	P (W vs C)
Calcium	87 ± 7.0	24 ± 3.2	P < 0.001
Magnesium	41.7 ± 2.3	27.6 ± 1.6	P < 0.005

Results are given as mean ± S.E. Units are nmoles/mg protein.

Abbreviations: WA: warm-acclimated; CA: cold-acclimated; IBAT: interscapular brown adipose tissue.

Table 8 also shows that the magnesium content of IBAT mitochondria from cold-acclimated rats was found to be only 70% of that of mitochondria from warm-acclimated rats. This finding was of interest because it supported the hypothesis that a lower amount of endogenous magnesium in mitochondria from cold-acclimated rats than in mitochondria from warm-acclimated rats might be responsible for both the enhanced second swelling and the reduced time of its occurrence which was described in Part A. Höser et al. (1976) determined that when 70-80% of liver mitochondrial magnesium was released into the incubation medium there was a marked increase in mitochondrial volume. If it is postulated that the first calcium-induced swelling rates represent a consequence of the uptake of calcium with subsequent loss of mitochondrial magnesium and the second swelling represents the large increase in mitochondrial volume which occurs when 70-80% of mitochondrial magnesium has been released, then it would be expected that this large volume change would occur sooner and to a larger extent in mitochondria isolated from cold-acclimated rats since only about 15-19 nmoles Mg^{2+}/mg protein (i.e. 70-80%) would have to be released for this volume change to occur while approximately 29-33 nmoles Mg^{2+}/mg protein would have to be released in mitochondria isolated from warm-acclimated rats.

Two important questions arose from these findings. 1. Was this difference in magnesium levels an adaptive change or a change due to cold stress? 2. If the magnesium content of

mitochondria from cold-acclimated rats is lower than those obtained from the warm-acclimated rat, which mitochondrial compartment (matrix or intermembrane space) is depleted? The following two sets of experiments attempt to answer these questions and are described in sections C and D.

PART C: TIME COURSE OF CHANGE IN MAGNESIUM CONTENT IN INTERSCAPULAR BROWN ADIPOSE TISSUE MITOCHONDRIA DURING ACCLIMATION AND DEACCLIMATION TO COLD

1. Purpose of the Experiment

Since the previous experiment had revealed that IBAT mitochondria from cold-acclimated rats had a lower magnesium content than mitochondria from warm-acclimated rats, it was of interest to determine whether this change represented an adaptive change or was an effect of acute cold stress. To obtain this information the time course of the decrease in magnesium content of IBAT mitochondria during acclimation to cold was followed as well as the time course of the increase in magnesium content during deacclimation to cold.

2. Description of the Experiment

Acclimation: Young male rats (150-200 g) were kept at room temperature (27°C ±1°C) for 1 week after their arrival. Rats were then divided into two groups; warm-acclimated (WA) which were left at room temperature and cold-acclimated (CA), placed in a cold room having a temperature of 4°C ± 1°C. The time course of magnesium depletion was followed by taking the rats out of the ~~cold~~ and warm rooms at specific times.

Deacclimation; Rats had been kept in the cold (4°C ±1°C) for 5 weeks before the start of the experiment. The mean weight of the rats at this time was 367 ± 5 g. After the 5 week period, cold-acclimated rats were divided into two groups. One group remained in the cold and the other group was put into the warm and is termed cold-acclimated returned to warm (CA→W). The time course of the increase in magnesium in IBAT mitochondria was followed by taking the rats out of the cold and warm rooms at specific times.

3. Results and Discussion

The results of the acclimation experiment (Table IX) indicate that it takes about 2 weeks before the magnesium content of warm-acclimated rats is significantly decreased by exposure to cold. The deacclimation experiment (Table X) revealed that it takes about 4 weeks for the magnesium content of cold-acclimated rats, which were returned to the warm, to be significantly increased. These results indicate that the observed changes in magnesium content which occur during acclimation and deacclimation to cold do not appear to be due to acute cold stress but represent a slow adaptive change.

The change in magnesium content observed in BAT mitochondria during acclimation to cold occurs at about the same time as the maximum change in the 32,000 polypeptide (i.e. 2 wks. Fig. 7) but slower than the increase in the binding of GDP and ADP (Fig. 6) during acclimation to cold which is apparent within the first hour of exposure to cold.

TABLE IX

Time Course of the Decrease in Magnesium Content of IBAT Mitochondria During Acclimation to Cold

	WA	CA	
Time (days)	nmoles Mg^{2+}/mg protein		
1	38.1 ± 6.7	39.2 ± 4.8	NS
3	31.6 ± 3.5	20.5 ± 2.4	NS
7	33.5 ± 2.0	26.6 ± 2.1	NS
14	46.4 ± 4.7	26.8 ± 1.7	< 0.01
28	42.2 ± 4.6	23.1 ± 1.3	< 0.0125

Young warm-acclimated (WA) rats were divided into two groups. One group remained in the warm and the other was put into the cold (CA). Rats from both groups were removed from their respective temperatures at the indicated times. It should be noted that these times do not necessarily indicate the sequence in which the experiments were performed. For example, the experiment in which the magnesium content was measured in rats which had been cold exposed for 3 days was not necessarily performed before the experiment in which the magnesium content was measured in rats which had been cold exposed for 7 days. This is also true of Table X. Isolation of IBAT mitochondria was as described in Methods 1 and the magnesium content of the mitochondria was measured as described in Methods 4. 3 WA and 3 CA rats were used for each of the indicated times.

TABLE X

Time Course of the Increase in Magnesium Content of IBAT mitochondria During Deacclimation to Cold

Time (days)	CA → W	CA	
	nmoles Mg^{2+}/mg protein		
1	16.8 ± 1.3	15.7 ± 2.2	NS
3	21.3 ± 1.2	18.7 ± 0.6	NS
7	15.8 ± 0.5	16.0 ± 1.7	NS
14	16.0 ± 0.8	11.7 ± 0.7	< 0.02
28	27.4 ± 0.4	19.0 ± 1.5	< 0.01
42	19.5 ± 1.23	15.2 ± 0.4	< 0.05

Rats were kept in the cold (4°C ± 1°C) for 5 weeks at which time they were divided into two groups. One group remained in the cold and the other group was put into the warm and is termed cold-acclimated returned to warm (CA → W). Rats from both groups were removed from their respective temperatures at the indicated times. 3 CA and CA → W rats were used for each of the indicated times. As in Table IX the indicated times do not necessarily indicate the sequence in which the experiments were performed.

PART D; INTRAMITOCHONDRIAL LOCATION OF MAGNESIUM LOSS IN INTERSCAPULAR BROWN ADIPOSE TISSUE MITOCHONDRIA

1. Purpose of the Experiment

Although it was shown that magnesium loss upon cold-acclimation and an increase in magnesium levels upon deacclimation were adaptive changes and not acute stress responses, more information concerning these changes was necessary so that some physiological significance could be ascribed to them. It was thus of interest to know whether the magnesium depletion in IBAT mitochondria of the cold-acclimated rat was due to loss from the intermembrane compartment, from the matrix compartment or from both of these sites.

2. Description of the Experiment

A total of 23 rats (19 WA & 4 CA) used in this study had lived about 1 month at their respective temperatures before the start of the experiment. Their mean weights were WA: 368 ± 6.0, and CA: 312 ±16.0 g.

The procedure for isolating IBAT mitochondria was essentially the same as described in Methods 1 except that 0.25 M sucrose and 1.0 mM HEPES pH 7.2 was used instead of 0.25 M sucrose for the final two mitochondrial washings. The final suspension was brought to a concentration of 10 mg mitochondrial protein/ml.

To 0.5 ml of the mitochondrial suspension was added 0.5 ml of 250 M sucrose, 1 mM HEPES, 0.05% serum albumin and 0.2 mg digitonin/mg mitochondrial protein (final concentrations). After

incubation and centrifugation (see Methods 6) two fractions were obtained. The "pellet fraction" contained mostly mitoplasts, while the "supernate fraction" contained the contents of the intermembrane space. Malate dehydrogenase and adenylate kinase were used as marker enzymes for the matrix and intermembrane space respectively. Optimum conditions would therefore yield high adenylate kinase activity and low malate dehydrogenase activity in the supernate fraction and low adenylate kinase activity and high malate dehydrogenase activity in the pellet fraction. The magnesium concentration of each fraction was then measured. To obtain 100% activity, 0.5 ml of the 10 mg/ml mitochondrial suspension was treated in the same way as described above except that the centrifugation step was omitted. The adenylate kinase activity and the malate dehydrogenase activity were then measured in this fraction which contained both mitoplast and intermembrane components.

3. Results and Discussion

That the fractionation procedure produced relatively uncontaminated samples of intermembrane space and of matrix is indicated by the low adenylate kinase activity in the pellet and the low malate dehydrogenase activity in the supernatant (Fig. 25 A & B).

The most striking observation from this study is the finding that there is only 5% of the total content of magnesium found in the intermembrane space in mitochondria of both warm-acclimated and cold-acclimated rats (Fig. 25 C). This is in contrast to the situation in liver and heart where 50% and 31% respectively of the total magnesium content was found in this compartment

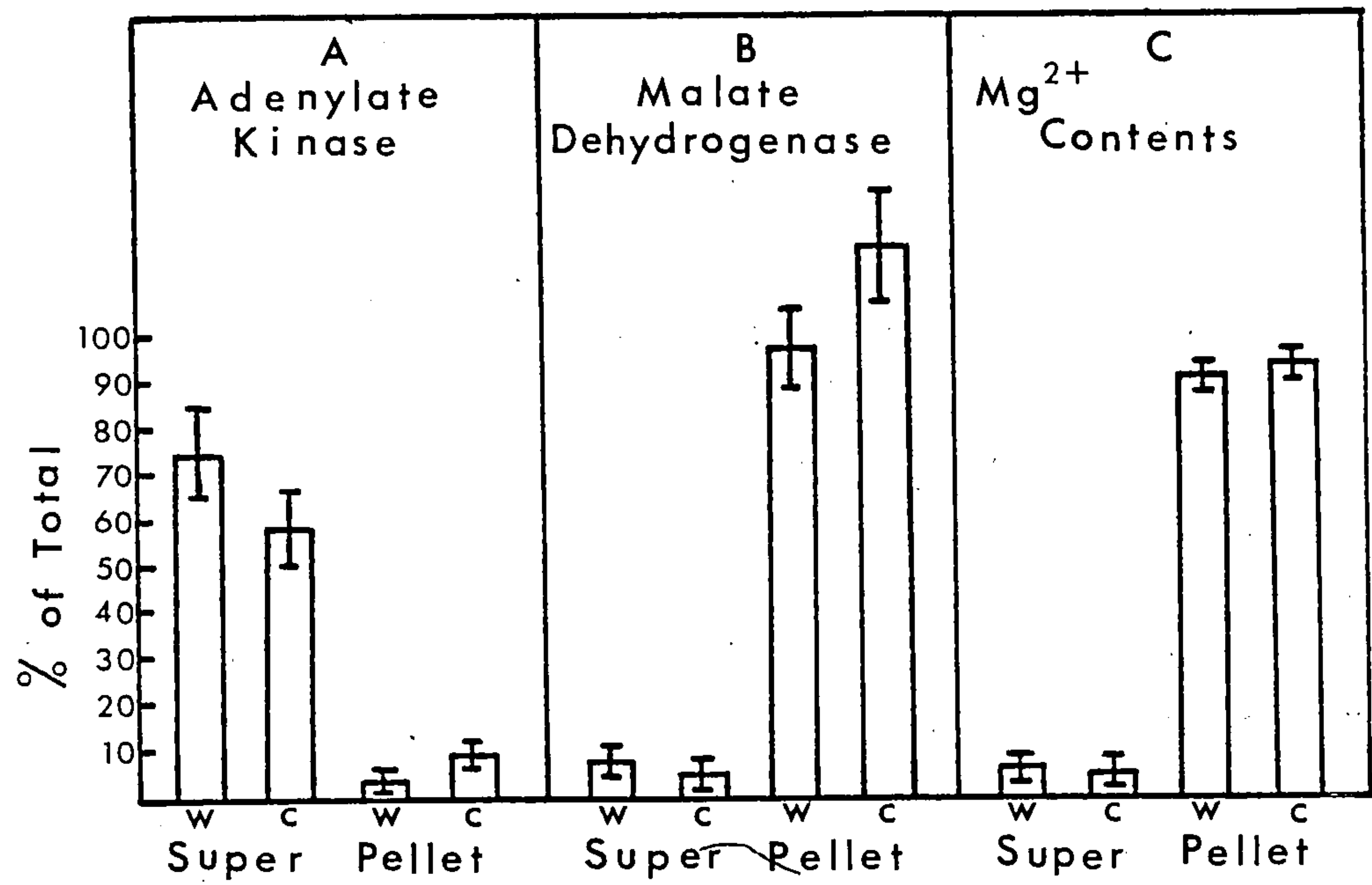

Figure 25. Enzyme Activities and Magnesium Contents of Digitonin Treated IBAT Mitochondria From Warm- and Cold-Acclimated Rats

The adenylate kinase activity (Fig. 25A) and the malate dehydrogenase activity (Fig. 25B) in the pellet and supernatant fraction is shown for digitonin treated IBAT mitochondria from warm (W)- and cold (C) - acclimated rats. The magnesium content for each of the two fractions is shown in Fig. 25C. All values are expressed as percentage of either total enzyme activity or total magnesium content of IBAT mitochondria. Total enzyme activity for adenylate kinase was 0.192 ± .036 μmoles NADPH/ml min mg protein and the total enzyme activity for malate dehydrogenase was 0.83 ± 0.095 μmoles NADH/ml min mg protein. Total magnesium content of warm - and cold-acclimated rats was 35.3 ± 3.2 and 20.2 ± 1.9 nmoles Mg^{2+}/mg protein respectively.

(Bogucka & Wojtczak, 1971). This finding raises the possibility that the magnesium binding proteins which were first described in liver mitochondria (Bogucka & Wojtczak, 1976) might not be present in the intermembrane space of IBAT mitochondria. In vivo it would be expected that any free magnesium in the cytoplasm would be able to diffuse across the outer membrane and enter into the intermembrane space. However, the absence of the magnesium binding proteins would imply that any change in the cytoplasmic level of magnesium would immediately influence the magnesium level in the intermembrane space. The consequences of such a flux is, however, not immediately apparent.

Since virtually all the mitochondrial magnesium was present in the matrix plus membranes compartment in both warm- and cold-acclimated rats it may be presumed that the depletion of mitochondrial magnesium seen in the cold-acclimated rat occurs in the matrix and membrane fraction.

PART E: CALCIUM-INDUCED SWELLING RATES OF LIVER MITOCHONDRIA ISOLATED FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

The changes observed so far in IBAT mitochondria of cold-acclimated rats are a decrease in the level of magnesium, lost primarily from its major location, the matrix compartment, and an apparently associated increased rate of swelling in response to stimulation by calcium. In order to determine whether the changes observed were related to the enhanced thermogenic activity of the IBAT or were related to some other change in the cold-acclimated rats, similar processes were studied in mitochondria from a tissue known not to undergo changes in thermogenic activity, the liver.

2. Description of the Experiment

A total of 6 rats (3 WA & 3 CA) had lived at least 2 months at their respective acclimation temperatures before the start of the experiment. The mean weights of the rats were WA: 564 ± 31, CA: 440 ± 17.

The procedure used was exactly as described in measuring calcium-induced swelling rates in IBAT mitochondria.

3. Results and Discussion

In contrast to IBAT mitochondria, there was no significant differences found in calcium-induced swelling rates and no second swelling was observed in liver mitochondria isolated from warm- and cold-acclimated rats (Fig. 26). However, the absence of an observed second swelling in liver mitochondria is most probably due to the much higher rates of swelling which occurred,

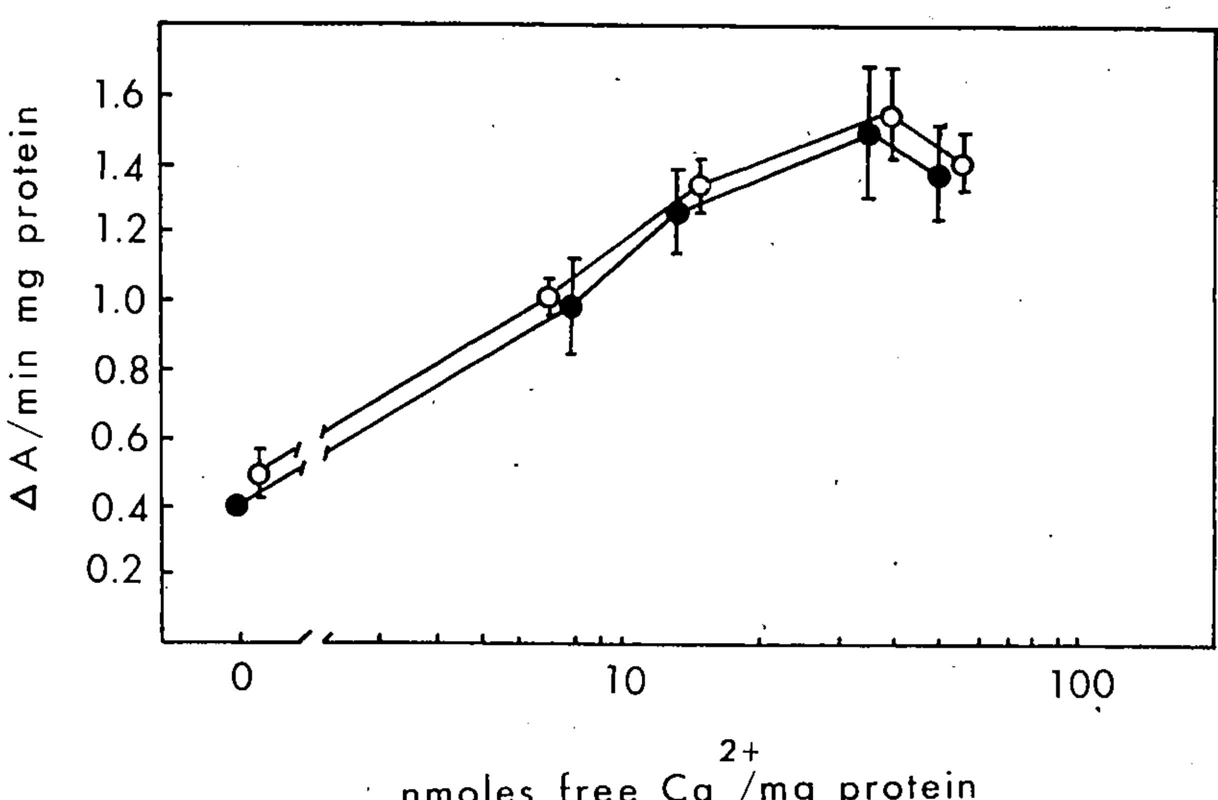

Figure 26. The Effect of Calcium Concentration on the Rate of Calcium-Induced Swelling in Liver Mitochondria

The figure shows rates of calcium-induced swelling as a function of free calcium concentration in liver mitochondria isolated from warm-acclimated [●] and cold-acclimated rats [o]. The incubation conditions for this study were the same as the conditions described for IBAT mitochondria (Fig. 20). Free calcium was present at the indicated concentrations. Temperature was 25°C and the reaction was started by the addition of $CaCl_2$.

both spontaneously and at all calcium concentrations studied. If one compares Fig. 26 with Fig. 21 it can be seen that the rate of swelling in liver mitochondria is about four times greater than that of IBAT mitochondria. These high rates of swelling in liver mitochondria might therefore be the reason why a second swelling was not observed. This explanation is supported by the observation that in one experiment using liver mitochondria in which an exceptionally low spontaneous rate of swelling occurred, a second swelling was observed although this result could not be reproduced.

The reason for the much higher rates of calcium-induced swelling in liver mitochondria as compared to IBAT mitochondria is not readily apparent. One possible explanation might be related to the extensive degree of condensation of the matrix volume when IBAT mitochondria are stored in sucrose since the oxidation of succinate is partially sensitive to this volume. Liver mitochondria do not appear to be as extensively condensed when stored in sucrose (Nicholls et al., 1972).

Since a difference was not found in rates of calcium-induced swelling in liver mitochondria isolated from warm- and cold-acclimated rats it was of interest to measure the magnesium levels of these mitochondria.

PART F: MEASUREMENT OF CALCIUM AND MAGNESIUM CONTENTS OF LIVER MITOCHONDRIA ISOLATED FROM WARM- AND COLD-ACCLIMATED RATS

1. Purpose of the Experiment

It had been shown that the higher rates of calcium-induced swelling in IBAT mitochondria obtained from cold-acclimated rats was associated with a lower magnesium content of these mitochondria. Since no differences were found in calcium-induced swelling rates in liver mitochondria it was of interest to measure the magnesium levels in these mitochondria to see if a comparison of these measurements either supported or conflicted with the finding obtained with IBAT mitochondria. Calcium levels in liver mitochondria were also measured for comparison purposes.

2. Description of the Experiment

A total of 6 rats (3 WA & 3 CA) had lived at least 2 months at their respective acclimation temperatures before the start of the experiment. The mean weights of the rats were WA: 564 $\pm$ 31, CA: 440 $\pm$ 17.

The procedure was exactly as described in the Methods.

3. Results and Discussion

As the results show (Table XI) the calcium content of liver mitochondria isolated from cold-acclimated rats was found to be significantly lower than the calcium content of liver mitochondria isolated from warm-acclimated rats but a significant difference in the magnesium content of these mitochondria was not observed.

TABLE XI

Calcium and Magnesium Contents of Liver Mitochondria

Liver (n=3)	WA	CA	P (W vs C)
Calcium	16.6 ± 0.78	11.3 ± 2.18	< 0.025
Magnesium	34.7 ± 2.55	31.5 ± 2.68	NS

The table shows the calcium and magnesium contents of liver mitochondria isolated from warm- and cold-acclimated rats. The homogenization and the first mitochondrial washing was performed using 0.25 M sucrose, 0.2 mM EDTA and 1.0 MM HEPES. 0.25 M sucrose was used for the remaining 2 mitochondria washings. The cation contents were measured exactly as described in Methods 4. Results are given as mean ± S.E. Units are nmoles/mg protein.

Abbreviations: WA: warm-acclimated; CA: cold-acclimated; NS: not significant; S.E. standard error.

The decrease in the calcium content in cold-acclimated rat liver mitochondria agrees with the findings of McBurney and Radomski (1973) who measured the calcium content of liver mitochondria obtained from warm- and cold-acclimated rats in the absence and presence of EDTA (Table VII). However, the calcium contents of rat liver mitochondria reported by McBurney and Radomski (1973) are only about one third the calcium contents of rat liver mitochondria shown in Table XI. On the other hand, the calcium content of liver mitochondria isolated from warm-acclimated rats reported in Table XI are in agreement with the results of Carafoli and Lehninger (1971) who reported that rat liver mitochondria isolated from warm-acclimated rats contain between 10 and 14 nmoles mg protein. The reason for this discrepancy is not known although it might be due to a difference in performing the decanting procedure after each mitochondrial washing since this procedure is known to affect the purity of the mitochondria - containing fraction (Johnson & Lardy, 1967).

The magnesium contents of liver mitochondria observed in this study are in the same range as those reported by McBurney and Radomski (1973). However, in contrast to their observed lower magnesium content in liver mitochondria obtained from cold-acclimated rats in the presence of EDTA (Table VII) no significant differences were detected in the magnesium content of rat liver mitochondria obtained from warm- and cold-acclimated rats. One possible reason for this is that McBurney and Radomski (1973) had used a higher concentration of EDTA than was used

in this study (1.0 mM EDTA vs 0.2 mM EDTA) so that if liver mitochondria isolated from cold-acclimated rats contained a more labile pool of endogenous magnesium than liver mitochondria isolated from warm-acclimated rats then the higher concentration of EDTA might remove a larger proportion of the magnesium in the former.

The finding that the magnesium content of liver mitochondria isolated from warm-acclimated rats is not significantly different from that of liver mitochondria isolated from cold-acclimated rats is compatible with the observation that rates of calcium-induced swelling in these mitochondria are also not significantly different. This result therefore supports the hypothesis that a significantly lower endogenous content of magnesium in IBAT mitochondria isolated from cold-acclimated rats is responsible for the enhanced rate of swelling observed in these mitochondria.

CONCLUSIONS

The purpose of the research reported in this thesis was to investigate the role, if any, of mitochondrial magnesium ions in nonshivering thermogenesis. This was done by measuring the endogenous magnesium level in brown adipose tissue mitochondria isolated from warm- and cold-acclimated rats to determine whether any differences exist and whether these differences, if found, might give a clue to the mechanism of nonshivering thermogenesis in brown adipose tissue of the cold-acclimated rat. Experiments were also performed using liver mitochondria since it is known that this tissue does not undergo changes in thermogenic activity during cold-acclimation. This was done in order to determine whether any changes in interscapular brown adipose tissue mitochondria following cold-acclimation were indeed related to the high thermogenic activity of this tissue observed in the cold-acclimated rat.

The major findings of the study were as follows:

1) IBAT mitochondria isolated from cold-acclimated rats display a greater rate of calcium-induced swelling than IBAT mitochondria isolated from warm-acclimated controls.
2) Although IBAT mitochondria isolated from warm- and cold-acclimated rats both undergo a second period of swelling which occurs to a greater extent at lower calcium concentrations than the first period of swelling, the second period of swelling occurs sooner and more rapidly in IBAT mitochondria isolated from cold-acclimated rats.

3) There is a decrease in the level of endogenous magnesium in IBAT mitochondria of cold-acclimated rats. The level reaches a minimum of after 1 to 2 weeks of cold exposure. The magnesium appears to be lost mainly from the matrix compartment. Approximately one month of deacclimation is required before the endogenous level of magnesium in IBAT mitochondria of a cold-acclimated rat increases again.
4) Only 5% of the total content of magnesium is located in the intermembrane space of IBAT mitochondria isolated from warm- and cold-acclimated rats.
5) Liver mitochondria isolated from warm- and cold-acclimated rats show no differences in calcium-induced swelling, do not display a second period of swelling, and have similar levels of endogenous magnesium.

The results indicate that the changes observed in IBAT mitochondria isolated from cold-acclimated rats are related to the enhanced thermogenic activity of this tissue since similar changes did not occur in mitochondria isolated from a tissue known not to undergo changes in thermogenic activity, namely the liver.

The question which now arises is how the observed alterations in calcium-induced swelling and in ion content which occur in IBAT mitochondria during cold-acclimation relate to the elusive biochemical mechanism of nonshivering thermogenesis. The slow decrease in magnesium content of IBAT mitochondria during acclimation of the rat to cold parallels in

its time-course the slow change in the composition of the mitochondria which leads to a larger proportion of a 32,000 polypeptide, a component of the thermogenic proteon conductance pathway (compare Figure 7 and Table X). It seems likely, therefore, that the decrease in magnesium level is related to the altered thermogenic functioning of the mitochondria. However, a role for a reduction in magnesium level is not immediately apparent. It might be involved in altered permeability of the mitochondrial membrane to other cations, and thus in a thermogenic cycling of cations.

The presence of only 5% of the total mitochondrial magnesium in the intermembrane space of IBAT mitochondria of both warm-acclimated and cold-acclimated rats suggests that the specific magnesium binding proteins of liver mitochondria, responsible for binding 50% of the total mitochondrial magnesium (Bogucka and Wojtczak, 1971; see Table VI), are not present in IBAT mitochondria. Since the function of these proteins is not understood, a role for their postulated absence in IBAT mitochondria is not apparent.

Interpretation of the increased rate of calcium-induced swelling in IBAT mitochondria from cold-acclimated rats is subject to the various assumptions about the meaning of decreases in absorbance during mitochondrial swelling (see discussion, Part B, section 4). It seems likely that the "first swelling" represents

matrix expansion and that the "second swelling" represents an increase in the overall size of the mitochondria (see Figure 17). The shortened time to the onset of the second swelling in IBAT mitochondria of the cold-acclimated rat is suggested to be associated with the lower magnesium content of these mitochondria. Swelling occurs when the calcium-induced loss of mitochondrial magnesium results in the low level of about 10 nmoles per mg of protein, and this is achieved sooner in the depleted mitochondria of the cold-acclimated rat. The increased rate of swelling may be due to increased permeability of the mitochondrial membrane associated with the reduced magnesium concentration. Another possibility would be an altered composition of IBAT mitochondrial membrane lipids in cold-acclimated rats. However, evidence is controversial about possible alterations in the composition of IBAT mitochondrial phospholipids in cold-acclimated rats (Cannon et al., 1975; Ricquier, Mory and Hemon, 1975) and this remains a subject for further investigation.

In conclusion, the results reported in this thesis indicate that mitochondrial magnesium may have a role in the control of the mechanism of nonshivering thermogenesis in IBAT mitochondria. Further work is necessary to establish the nature of this role.

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APPENDIX

Calculation of the Rate of Calcium-Induced Swelling

The rates of the first swelling (Fig. 21) were obtained for each of the indicated calcium concentrations by drawing a tangent to the decrease in absorbance (referred to as Rate #1 in Fig. 20) and measuring its slope. For example, according to Fig. 21 the slope of the tangent drawn from Rate #1 in Fig. 20, which is representative of the events which occurred at 50 nmoles calcium/mg protein, in cold and warm-acclimated rats were 0.3 and 0.25 ΔA/min mg protein respectively. Similarly, the rates of the second swelling (Fig. 22) were obtained for each of the indicated calcium concentrations by drawing a tangent to the decrease in absorbance (referred to as Rate #2 in Fig. 20) and measuring its slope. For example, according to Fig. 22, the slope of the tangent drawn from Rate #2 in Fig. 20 in cold and warm-acclimated rats were 0.7 and 0.4 ΔA/min mg protein respectively. The rates of calcium-induced swelling in liver mitocondria (Fig. 26) were also obtained in this manner.