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ENZYMIC AND METABOLIC PROFILES OF THE ALTERED MITOCHONDRIA IN SKELETAL MUSCLE OF COLD-ACCLIMATED RATS

by

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TO NASSER AND KHADIJAH

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ABBREVIATIONS

ST	-	shivering thermogenesis
NST	-	nonshivering thermogenesis
SNS	-	sympathetic nervous system
T_4	-	thyroxine
T_3	-	triiodothyronine
ADP	-	adenosine-5'-diphosphate
ATP	-	adenosine-5'-triphosphate
Pi	-	inorganic phosphate
Cyclic AMP	-	adenosine-3', 5'-monophosphate

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ABSTRACT

Nonshivering Thermogenesis (NST) is an adaptation in which there is a high metabolic rate that can be switched on and off according to the needs of the animal. The mechanism of NST is still not understood. Since the skeletal muscles are believed to be the major site where NST occurs, and since NST is a condition in which there is a high metabolic rate, then, changes in the mitochondria of this tissue are expected. Thus the aim of this work was to look for specific biochemical changes in the skeletal muscle mitochondria of cold-acclimated rats that might shed some light on the mechanism of NST in this tissue. The following characteristics were studied:

1 - The respiratory and oxidative properties of the isolated skeletal muscle mitochondria of both warm- and cold-acclimated rats. The results showed that the isolated skeletal muscle mitochondria of cold-acclimated rats have normal ADP/O ratios and normal respiratory ratios. However, the results showed that these mitochondria have higher rates of respiration in both states 3 and 4, the former being more marked.

2 - The levels of some enzymes of the Kreb's cycle, and related enzymes. Changes were found in the specific activities of the mitochondrial α-glycerophosphate and malate dehydrogenases, the former increased by 60%,

and the latter decreased by 24%.

3 - The transport of reducing equivalents from cytosol into mitochondria. The results showed that the α-glycerophosphate shuttle might have a greater capacity in the skeletal muscles of cold-acclimated rats leading to a greater capacity for glycolysis.

4 - The transport of ADP from cytosol into mitochondria. The results showed a 35% increase in the rate of ADP uptake by the isolated skeletal muscle mitochondria of cold-acclimated rats.

The significance of all the above findings are discussed as well as the possible involvement of the thyroid hormones in the mechanism of NST.

CHAPTER A

INTRODUCTION

Cold acclimation is a problem which has always fascinated scientists; the scientific dream being the expansion of the habitat of the human race to the cold regions of the earth with the use of the least amount of energy such as heating fuel. Until recently, the problem has been dealt with only in physiological terms. This is not surprising since cold acclimation is the net outcome of whole body adaptation includ--ing hormonal, nervous, cardiovascular and other physiological changes. However, recently the great advance of biochemical methods and techniques has sparked the beginning of a very active study of cold acclimation and the processes behind it at the molecular level.

In this work an attempt has been made to push present knowledge about the molecular mechanism of cold acclimation a step forward. Since the mitochondrion is generally accepted to be the powerhouse of the cell, the study has been mainly concentrated on this organelle. Also, since it was impractical to study the mitochondria of all tissues , one tissue which is believed to be the major site for heat production during cold acclimation, namely skeletal muscle, has been chosen.

Therefore in this thesis, comparative studies on the mitochondria of skeletal muscle of cold and warm-acclimated rats are described in order to find out those changes which

make the acclimation to cold possible.

In view of the results of this work and the discussion presented in the general conclusion, it is necessary to introduce the reader to some areas which will help him comprehend the general problem of cold-acclimation, the significance of the findings and the validity of the general discussion.

Thus, the introduction will start with a classification of the general defence mechanisms which a homeothermic animal has when put in the cold, and which then make his stay in the cold possible. This will be followed by a discussion of why skeletal muscle is thought to be the major site of heat production in the cold. Then, the roles of the sympathetic nervous system and the thyroid hormones in the adaptation to cold will be discussed. This part of introduction is very important for the understanding of the general conclusion. This will be followed by a summary of the present knowledge of the molecular mechanisms which make living in the cold possible. Lastly, an attempt will be made to review the known changes in skeletal muscle mitochondria under different sets of conditions. This last part of the introduction is intended to give the reader an insight into the flexibility of mitochondria in terms of the many ways they can change and adapt to the external and internal environments.

SECTION I

SHIVERING THERMOGENESIS AND NONSHIVERING THERMOGENESIS (NST)

When an animal is put in a cold environment, the thermal gradient between the exterior and his body temperature increases, thus leading to an increased heat loss from the body. The body temperature therefore would tend to decrease if it were not for the presence in homeotherms of defence mechanisms which do the following:

a) Physiological adaptations which tend to decrease heat loss from the body.

These include vasoconstriction of blood vessels in the skin and piloerection.

b) Muscular shivering, which is controlled by the central nervous system and activated by cold.

This muscular shivering, which is not doing work, is known as shivering thermogenesis (ST) because it is essentially a heat-producing mechanism. The hydrolysis of the ATP during shivering liberates great amounts of heat which if continued can compensate for the greater loss of heat from the body. This means that ATP must be synthesized continuously at a faster rate. This in turn requires more substrates to be oxidized. That is why in the cold, animals eat more, and their sympathetic nervous system, which is activated by the cold, mediates a greater release of glucose from the liver

and free fatty acids from the white adipose tissue into the blood in order to be used by the tissues, especially the shivering muscles (for reviews, see Himms-Hagen, 1972 a and b).

c) The activation of mechanisms of heat production other than shivering called nonshivering thermogenesis (NST).

This NST replaces gradually shivering thermogenesis until the animal becomes totally dependent upon NST in order to produce the extra heat required to keep his body temperature constant. When this happens, the animal is considered to be cold-adapted to the external temperature. If the external temperature is further dropped, shivering might start again for some time until NST is further augmented. Thus, shivering thermogenesis can be described as the first line of defence until the second line, namely NST, is well developed.

The extent of the ability to develop NST is species dependent. It occurs in some newborn species (rabbit, guinea pig, rat, human), in hibernating mammals (bat, hamster, ground squirrel), and in species acclimated to cold. Thus, NST stays dormant until it is needed by the animal (Depocas, 1960b; and Himms-Hagen, 1970).

SECTION 2

THE LOCALIZATION OF THE MAJOR SITE OF NST

All tissues of the body are producing heat as a result of basal metabolism. Even in this case, the relative contribution of each organ to the total heat production is not known. During acclimation to cold, extra heat should be produced by the mechanism(s) of NST. A major problem to solve then is: which site of the body is the most extensively involved in the production of extra heat by NST. This problem is basic to the study of the mechanism of NST. One must know where it occurs, or at least where most of it occurs because the study of the mechanism of NST is a comparative one. One looks for changes in the cold-acclimated state and from these changes, expects to unravel the mechanism of NST.

In the literature, there are conflicting results on the site of heat production in NST. The controversy concerns three sites:

a) The visceral organs including the liver, the intestines and the kidneys.

b) The skeletal muscles.

c) The brown adipose tissue.

The most direct technique that can give information about the quantitative contribution of each of the above sites to NST is to measure the blood flow to each organ, and from the arteriovenous difference in oxygen content (the extraction of

oxygen from the blood), one can calculate the relative quantitative contribution of each organ. However, this method is practically impossible in small animals since cannulations of small vessels under physiological conditions cannot be done. Therefore, other methods must be used.

Based on the assumption that the cytochrome oxidase activity of an organ is an indication of its metabolic capacity, Jansky estimated that the skeletal muscles of the rat could contribute 50-70% of total NST (1966); the skin not more than 6% (1966); the kidneys, 5% (1966); the liver, less than 25% (1971, 1973). It must be noted however, that Jansky's definition of NST included the basal metabolic rate (1973).

The experiments performed by Jansky and Hart (1968), in which metabolic capacity was calculated from the blood flow through various organs of warm and cold-acclimated rats, showed that the visceral organs can account for the greatest proportion of the total NST. However, these experiments can be questioned for the following reason: the metabolic contributions of the visceral organs to NST were calculated by multiplying the increased blood flow to each organ in the cold-acclimated state by the concentration of oxygen in the blood. Therefore, a big assumption was made here, namely that the organs will consume all the oxygen of the extra blood that flows through them. In my view, this is an invalid assumption. However, even on the basis of such calculations, the authors estimated that the metabolic contribution of skeletal muscles to NST is 50% at most.

On the other hand, the results of Depocas (1958) argue strongly against the idea that the visceral organs are the major site for NST. Depocas performed experiments in which he restricted blood flow to the visceral organs of cold-acclimated rats and then exposed the rats to the cold. The rats showed a large increase in oxygen uptake similar to the sham-operated rats. In the later work, Depocas (1960 a) showed that the functionally eviscerated cold-acclimated rats reacted to the infusion of noradrenaline with an increase in oxygen consumption equivalent to that of the sham-operated animals. The conclusion of Depocas was that the visceral organs are not required for the switching on of NST, and that extravisceral tissues are quantitatively more important sites for NST. Another conclusion from the above experiments of Depocas is the following: If NST which is already developed in the cold-acclimated rat is localized in the visceral organs, then the infusion of noradrenaline or the cold exposure of the functionally-eviscerated rats could not have resulted in a response as great as that of the sham-operated rats. The response must have come from extravisceral tissues where NST is localized, probably the skeletal muscles.

Jansky and Hart (1963) were able to prove directly that skeletal muscles contribute to NST. They calculated the oxygen consumption of the muscles of the hind leg of the cold-acclimated rat, from blood flow values and the arterio-venous differences

in oxygen contents, before and after cold exposure or noradrenaline infusion. The results showed that the oxygen consumption increased more than two-fold. Although this finding does not prove that the skeletal muscles are the major site of NST, yet it proves two things: i) that the skeletal muscles contribute to NST, ii) that the skeletal muscles have the power to be the major site of NST.

Consistent with these statements (i and ii), are the findings of Mejsnar and Jansky (1971). The authors showed that the metabolism of the gracilis muscle is directly stimulated by noradrenaline, and not by increases in blood flow or increases in the amounts of substrates released under physiological conditions into the blood.

Three lines of findings from our laboratory are consistent with the proposal that skeletal muscles are the major site of NST. They are the following:

a) There is an increased turnover of some proteins of the skeletal muscle mitochondria, and none for the liver or kidney in cold-acclimated rats (Himms-Hagen et al, 1972).

b) The inhibition of mitochondrial protein synthesis in skeletal muscle by oxytetracycline prevents the development of the enhanced response to noradrenaline during acclimation to cold and reverses it if it has already developed (Himms-Hagen et al, 1975).

c) There are mitochondrial changes in size and number in the skeletal muscles of cold-acclimated rats. The mitochondria of

the skeletal muscles of cold-acclimated rats increase in number and decrease in size (Behrens and Himms-Hagen, in press).

Although the brown adipose tissue hypertrophies in cold-acclimation and becomes the warmest region of the body (Himms-Hagen, 1970), its contribution to the total NST can be considered to be small (between 6 and 12%, Himms-Hagen, 1969). In addition, although the calculations of Jansky and Hart (1968) which were based on blood flow and the total extraction of oxygen from the blood were criticized, yet this method can be used to eliminate quantitatively some of the organs as significantly contributing to total NST, including the brown adipose tissue. The authors estimated that the brown adipose tissue could contribute to only 6% of the total oxygen consumption of the rat.

In experiments aimed to estimate the quantitative contribution of the brown adipose tissue to NST, the surgical removal of the interscapular brown adipose tissue (about one third of the total brown adipose tissue in the cold - acclimated rat) has had relatively little immediate effect upon the enhanced calorigenic response to noradrenaline in cold-acclimated rats (Himms-Hagen, 1969; and Foster, 1974), but in a number of independent studies, it has caused a delayed reduction of 40 - 60% after two to four days (Hayward and Davies, 1972; Himms-Hagen, 1969; Horwitz et al, 1972, and Leblanc et al, 1972). However, two recent studies have shown no such delayed effect (Flattery and Sellers, 1972; and Foster, 1974).

These discrepancies were attributed to failure to monitor body temperature in the operated animals during noradrenaline infusion (Foster, 1974).

In conclusion, it is probably safe to consider the skeletal muscles as the major site of NST.

SECTION 3

THE ROLE OF THE SYMPATHETIC NERVOUS SYSTEM (SNS) IN COLD-ACCLIMATION.

The role of the SNS in cold-acclimation is best explained by discussing it under the following headings:

i) The SNS is activated during cold-exposure and cold-adaptation.

ii) The catecholamines can mimic the switching on of NST by cold exposure.

iii) The adaptation to cold requires the presence of an intact SNS.

iv) The SNS is essential for the mobilization of the energy reserves.

v) The calorigenic action of the catecholamines.

Each part will now be discussed separately:

i) The SNS is activated during cold-exposure and cold-acclimation.

The time-course of changes in the excretion of the catecholamines after cold-exposure was studied by Leduc (1961). He found that during the first week of cold exposure, the excretion of noradrenaline increases 5 times, after which it falls, but remains at high levels for several weeks until the rat becomes cold-acclimated. After that, the excretion falls to a slightly higher value than that of warm acclimated rats. On the other hand; the excretion of adrenaline follows a different pattern. It increases gradually and reaches its

maximum in about one week after which it falls in a few days to normal levels. Leduc (1961) also showed that the amount excreted is proportional to the severity of the cold exposure, and is greater in young animals (age-dependent). On the other hand, during deacclimation (the removal of the rat from the cold), the excretion of noradrenaline returns to normal within one or two days. The increase in the excretion of noradrenaline in the cold is due to an increase in its secretion rather than a decrease in its degradation since Shum et al., (1969) showed that there is also an increase in the excretion of the major metabolites of the catecholamines. Therefore, in cold-exposure and cold-acclimation, there is an increase in the synthesis and secretion of the catecholamines, especially of noradrenaline.

ii) <u>The catecholamines mimick the switching on of NST by cold-exposure</u>.

The intravenous infusion of the catecholamines results in an increase in the oxygen consumption of the warm-acclimated rat, and a much greater increase when the animal is cold-acclimated (Hsieh and Carlson, 1957; Himms-Hagen, 1970). When the infusion is stopped, the oxygen consumption returns to normal in both warm and cold-adapted rats. The time-course of the increase in the calorigenic response to the catecholamines during the 4 week period required for cold-adaptation, coincides with the decrease in shivering. The response becomes maximal when shivering becomes minimal (Depocas, 1960b). Especially

interesting are the results of Leblanc and Pouliot (1964) who showed that the resistance to cold increases when rats are treated with noradrenaline for long periods. Also, the calorigenic respose to noradrenaline is enhanced. Therefore, the SNS can switch on and off NST, and plays a role in the development of NST.

iii) <u>The adaptation to cold requires the presence of an intact SNS</u>:

This can be proved by deactivating the SNS in some way and studying whether the animal can still adapt to cold or not. This can be done by surgical, immunological and pharmacological techniques. The conclusion drawn from such experiments was that an intact SNS is required for cold-adaptation. These findings were reviewed by Himms-Hagen (1975).

iv) <u>The SNS is essential for the mobilization of the energy reserves</u>:

The first action of the catecholamines on the intact animal in terms of the mobilization of the energy reserves is their hyperglycemic effect. This response is mediated by an increase in liver glycogenolysis, an increase in liver gluconeogenesis, and inhibition of insulin secretion leading to decreased utilization of glucose by the tissues (see review by Himms-Hagen, 1967). The second major metabolic effect of the catecholamines on the intact animal is the rise in blood free fatty acids concentration due to an increased liberation of these compounds from the white adipose tissue. This effect

results from an increase in the rate of lipolysis in the white adipose tissue. The catecholamines also act on other tissues to mobilize their own reserves for internal use. Examples are the skeletal muscles which use their gylcogen, and the brown adipose tissue which uses its triglycerides. Therefore, the catecholamines mobilize the energy reserves for internal and external use by the tissues, thus supplying the substrates required for the eventual increase in metabolic rate. The mechanism of this action of the catecholamines is believed to be due to an increased concentration of cyclic AMP which in turn stimulates the pathways which lead to the eventual response.

v) The calorigenic effect of the catecholamines.

The mechanism of the calorigenic action of the catecholamines is not understood. The increase in substrate concentration due to the increased mobilization of the energy reserves is not the factor which controls the metabolic rate. The metabolic rate is controlled by the phosphorylation state ratio (ATP)/(ADP) (Pi) (Wilson et al., 1974).

During cold-exposure of the warm-acclimated rat, the signal for the beginning of the increase in oxygen consumption is the rise in ADP production in the shivering muscles as a result of the utilization of ATP for the contractile process. The nature of the signal in cold-acclimation is not known. There are at present two major hypotheses which try to explain the calorigenic effect of the catecholamines in cold-acclimated

rats:

A - The electron transport becomes less restrained by the availability of ADP in some way.

B - An increase in the role of energy - consuming pathways and/or processes.

These will be discussed in greater detail under section 4.

SECTION 4

THE MECHANISM OF NST, CURRENT CONCEPTS

The mechanism of NST can be described as a silent mechanism whose aim is to produce heat in order to compensate for the great loss of heat by the animal to the exterior. It is described as silent because it does not involve such actions as shivering (during cold exposure), or contraction (during exercise). Since the silent mechanism of NST results in an increase in oxygen consumption of the animal in the cold, then the only way possible to study this mechanism is to put down all the possible hypotheses that theoretically can lead to an increased oxygen consumption, and then test each separately. This mechanism must fulfill two requirments: i) It must be "switched on" by the catecholamines; ii) It must occur in tissues where NST occurs and not occur in tissues where NST does not occur. There are, at present two major hypotheses which can explain an increase in oxygen consumption. They are the following:

A - The electron transport becomes less restrained by the availability of ADP.

B - An increase in the operation of energy-consuming pathways and/or processes.

Each hypothesis will now be discussed separately.

Hypothesis A:

In normal mitochondria, electron transport cannot occur

to an appreciable extent unless ADP is present. If this locked relationship becomes weakened in some way, electron transport will then proceed less restrained by the availability of ADP. This situation is known as uncoupling or loose coupling and an example of this type has been reported in the isolated mitochondria of the brown adipose tissue (Flatmark and Pedersen, 1975). When the mitochondria of the brown adipose tissue are isolated by methods used to isolate mitochondria of other tissues, they are found to be uncoupled, in the sense that the addition of ADP does not stimulate the respiration. Originally, two groups of investigators, each using different methods, formulated two different mechanisms to explain the uncoupled state of the brown adipose tissue mitochondria. The first group used ATP and carnitine in the incubation medium (Hittelman et al., 1969), and were able to bring the mitochondria into a coupled state. This group suggested that because of the high concentration of free fatty acids in the brown adipose tissue, and due to the high sensitivity of the brown adipose tissue mitochondria to free fatty acids, a minor fraction of these fatty acids controls the state of loose coupling in the mitochondria. The removal of a small fraction of these fatty acids (by ATP and carnitine) can bring the mitochondria from the uncoupled to the coupled state (Cannon, 1971). The second group of investigators used albumin and ATP in the incubation medium to bring the uncoupled

mitochondria to the coupled state (Hohorst and Rafael, 1968). This group suggested that changes in the relative concentrations of adenine nucleotides within the mitochondria may be part of a mechanism regulating coupling (Pedersen and Grav, 1972; Skaane et al., 1972). It was suggested that the nucleotides could act on the energy-conserving mechanism of the mitochondria by some conformational change in the mitochondrial membrane. Later, the above two mechanisms were reconciled by the hypothesis of Christiansen (1973). He proposed that the free fatty acids are not only causing loose coupling, but by way of the acyl CoA formed from them in the cytoplasm (and which requires ATP for its synthesis), inhibiting the adenine nucleotide translocase of the inner mitochondrial membrane. The resulting change in the relative mitochondrial adenine nucleotide concentration also contributes to the regulation of coupling. Thus, the free fatty acids in the brown adipose tissue play three roles:

i) Act as substrates to support the high metabolic rate.

ii) Regulate the state of coupling.

iii) Change the relative adenine nucleotide concentrations in the mitochondria via inhibition of the adenine nucleotide translocase. This change will then act in collaboration with the fatty acids to control the coupled state.

This mechanism of uncoupling in the brown adipose tissue

mitochondria is a dynamic one in the sense that the degree of uncoupling is a process that gradually develops during cold-acclimation and reverts slowly to normal upon deacclimation (Andersen et al., 1970).

Hypothesis B:

This hypothesis is even more complicated than hypothesis A since one can think of a multitude of energy-consuming pathways and processes. For some energy-consuming processes, it is not certain whether ATP is the energy donor. Therefore, this hypothesis can be divided into two parts:

Part 1: Those energy-consuming pathways and/or processes where ATP is the energy donor.

Part 2: Those energy-consuming processess where ATP might not be the energy donor.

I will now discuss each part separately:

Part 1 can be further divided into 3 subparts:

i) subpart a: synthetic and degradative pathways.

ii) subpart b: futile enzymic cycles.

iii) subpart c: the Na K ATPase

The synthetic pathways (subpart a) are those for triglyceride, glycogen, urea and glucose. The reason why these synthetic pathways are suspected to play a role in consuming ATP, was the fact that the catecholamines deliver great amounts of substrates to these pathways. Calculations of the theoretical contribution of such synthetic pathways

to the total increase in oxygen uptake after infusion of catecholamines into warm-acclimated rats, showed that the contributions fall short of explaining the large increase in oxygen consumption (Himms-Hagen, 1967). However, there exists the possibility that there are intracellular increases in the turnover of these synthetic pathways which are not included in plasma studies (Himms-Hagen, 1967).

The futile enzymic cycles (subpart b) are pairs of enzymes where the end-product of one is the substrate of the other, and where both enzymes work together for a specific reason, namely, to consume ATP. The difference between the futile enzymic cycles and the synthetic and degradative pathways (subpart a) is that the former can be very easy to operate and control since they involve only two enzymes rather than two sets of enzymes. Examples of such futile enzymic pathways are: i) the glucokinase-glucose-6-phosphatase pair shown to occur in rat liver (Hue and Hers, 1974); ii) the phosphofructokinase-fructose diphosphatase pair shown to occur in kidney cortex (Newsholme and Underwood, 1966), and liver (Williamson et al., 1968). In addition, an increased operation of this futile cycle for the generation of heat was reported in the flight muscle of bumblebees during the warm-up before flight (Clark et al., 1973a,) and in the skeletal muscles of halothane-induced hyperpyrexia in pigs (Clark et al., 1973b). In fact, Williamson et al., (1971) suggested that this futile

cycle plays a role in NST in liver.

An increased activity of Na K ATPase (subpart c) was proposed to be a major component of the mechanism of NST (Horwitz, 1975). This topic is reviewed by Himms-Hagen (1976), and will be summarized briefly here. The proposition that this enzyme plays a major role in NST stemmed from the following lines of evidence:

i - Noradrenaline causes variable changes in the state of polarization of the cell membranes of various tissues leading to alterations in the ionic environments and thus to the stimulation of the enzyme (Horwitz, 1975).

ii - The activity of the enzyme increases in brown adipose tissue homogenates upon the addition of noradrenaline (Horwitz, 1975). In addition, the enzyme's activity doubles in liver slices of cold-acclimated rats, accompanied by a 30-80% increase in the rate of oxygen consumption (Videla et al., 1975).

iii) Ouabain (an inhibitor of the enzyme), inhibits the calorigenic effect of noradrenaline on brown adipose tissue (Pain and Jacobs, 1973). In addition, the skeletal muscles of cold-acclimated hamsters (Horwitz, 1975), mice (Stevens and Kido, 1974), and the liver of cold-acclimated rats (Videla et al., 1975), were shown to have an increased ouabain-sensitive respiration.

It must be noted however, that one should be cautious in interpreting this evidence for the following reasons:

i - Almost all hormones cause changes in the state of polarization of the cell membranes, and yet most do not have calorigenic effects.

ii - The activity of Na K ATPase seems to increase in other states of hypermetabolism such as hyperthyroidism (Ismail-Beigi and Edelman, 1971, 1974), and ethanol feeding (Israel et al., 1973). Thus it is not specific to cold-acclimation.

iii) - Ouabain has effects other than inhibiting the Na K ATPase (see Himms-Hagen, 1976). Examples are the following:

1. It inhibits lipolysis in white adipose tissue.
2. It stimulates gluconeogenesis in kidney.
3. It inhibits gluconeogenesis in liver.
4. It has insulin-like effects on glucose uptake by muscle and white adipose tissue.
5. It modifies Ca^{2+} transport.

Thus respiration may be altered by ouabain because of any of these effects.

iv - An increased ouabain-sensitive respiration of liver slices also occurs in warm-acclimated rats treated with adrenaline (Bernstein et al., 1975). This finding implies that there are reserve pump sites not in use un the cells. These dormant pump sites can be unmasked by cold-exposure or by adrenaline.

Part 2: Those energy-consuming processes where ATP might not be the energy donor.

This has been the least defined and studied. Some authors have included this part under the uncoupling hypothesis, and considered it a kind of uncoupling since some high-energy compounds synthesized along the pathway of oxidative phosphorylation are consumed, and thus ATP is not synthesized. However, I have defined loose coupling as that state where electron transport becomes less restrained by the availability of ADP in some way, and not as that state where electron transport becomes uncoupled from ATP synthesis. This part can be also divided into the following subparts:

i - subpart a: ion pumping

ii - subpart b: cellular and organelle movements.

Ion pumping (subpart a) by cells and organelles is a process that requires energy. The most studied ions are Ca^{2+} and K^{+}. The mitochondria, for example, will spend energy for taking up Ca^{2+} in preference to ADP phosphorylation (Lehninger, 1974). More studies are required in the field before any conclusion could be made about the significance of ion pumping in thermogenesis.

Cellular and organelle movements (subpart b) could play a role in thermogenesis. In vivo, cells and their organelles are in a state of continuous motion. Organelles change their shape and move from one part of the cell to another. These

active movements are most noticed during cell division. In fact, recent electron-microscopy studies have shown that most cells contain actin and myosin filaments forming complex networks (Marx, 1975).

The possibility exists that an increase in such movements might constitute a thermogenic process that contributes to NST (Himms-Hagen, 1976).

SECTION 5

THE ROLE OF THE THYROID HORMONES IN COLD-ACCLIMATION

The best way of discussing the role of the thyroid hormones in cold-acclimation is to divide it into the following parts:

Part 1 - The thyroid hormones are necessary for the successful survival in the cold.

Part 2 - The modes of secretion and utilization of the thyroid hormones change after cold-exposure and during cold acclimation.

Part 3 - The thyroid hormones - catecholamines interrelationships.

Part 4 - The calorigenic mechanism of the thyroid hormones.

Part 1 - The thyroid hormones are necessary for the successful survival in the cold.

Hsieh (1962) showed that when rats were fed with an iodine-deficient diet and an antithyroid drug for 4 weeks before exposure to 4° C, they died in one day. However, when the rats were fed with commercial diet and antithyroid drug, they were able to live in the cold for 17 days. The conclusion was that the iodine present in the commercial diet allowed the rats to survive for only 17 days in the cold. The thyroid gland could still synthesize some thyroid hormone using the iodine present in the commercial diet even in the presence of

the antithyroid drug. On the other hand, Hsieh (1962) found that the injection of thyroid hormones prevented death, and the growth of the rats became similar to that of the control rats. His conclusion was that the rats cannot survive in the cold without some circulating thyroid hormone. Hsieh (1962) also showed that the cold-acclimated rats can tolerate doses of T_3 that are toxic to the warm-acclimated rat, and his conclusion was that the cold does not increase the sensitivity of the rat to the thyroid hormones. Another conclusion from this finding is that changes have occurred in the body tissues so that they can utilize the high levels of T_3 with no drastic effects.

Essentially the same results were obtained by other authors (Leblond and Gross, 1943; Sellers and You, 1950; Sellers et al., 1951). Their results showed that athyroid rats cannot survive in the cold, and that the injection of thyroxine can make the survival in the cold possible. Therefore, the conclusion made by all the above authors was that some thyroid hormone is indispensable for prolonged survival in the cold.

Part 2 - The modes of secretion and utilization of the thyroid hormones change after cold exposure and during cold-acclimation.

It is generally accepted that the thyroid gland is activated after cold-exposure and cold-acclimation. Different

authors came to this conclusion by observing different things. For example, cold has been shown to increase the storage (D'Angelo, 1960) and secretion (Knigge, 1960) of the pituitary thyroidotrophic hormone. Other authors reported an increase in the weight of the thyroid gland (Stevens et al., 1955), an increase in the acinar epithelial cell height of the thyroid gland (Dempsey and Astwood, 1943) an increase in radioiodine thyroid output (Brown-Grant, 1956) and an increase in the peripheral degradation of the thyroid hormones (Cottle and Carlson, 1956; D'Angelo, 1960; Gregerman, 1963; Galton and Nisula, 1969).

It must be mentioned, however, that some authors argue against the involvement of the thyroid hormones in the mechanism of cold-acclimation since an increase in the thyroid stimulating hormone (TSH) levels in the plasma in response to cold was demonstrated only in rats and human infants (Varis et al., 1974), and not in other species. This argument however may be disputed since we know that the final availability of thyroxine at its target sites in the cells depends on such factors as the rate of degradation of the hormone in the tissues and the degree of binding of the hormone to the plasma proteins. In the guinea pig for example, Yamada et al., (1969) showed that the binding affinity of thyroxine to the plasma proteins decreased in the cold-exposed pigs, implying that in the cold, more free thyroxine is available to the tissues. In fact, this result is consistent with the results

of Ershoff and Colub (1950), and Heroux and Petrovic (1969) who reported a decreased concentration of serum-protein-bound-iodine after cold-acclimation. The decrease in the binding of thyroxine to the plasma proteins results in less detection of iodine in the protein fraction of the plasma of cold-acclimated rats.

However, the picture is not that simple. Balsam, (1974) showed that in cold-acclimated rats, there is a decrease in plasma protein-bound iodine, and yet, the relative binding of the plasma hormone (T_4 and T_3) to the plasma proteins increased. The explanation given to this discrepancy was that the thyroid gland in cold-acclimation was secreting T_3 in preference for T_4. Since T_3 is known to bind to plasma proteins one-sixth as tightly as T_4, then less iodine will be detected in the protein fraction of the plasma. In fact, this was found to be the case by Reichlin et al., (1973) who showed that plasma T_4 levels were less in cold-adapted rats, while plasma T_3 levels were higher than the controls. Bernal and Rey (1975b) showed that these changes were due to an increased conversion of T_4 to T_3 by the extrathyroidal tissues and not to a preferential secretion of T_3 by the thyroid gland.

On the other hand, since the binding of T_3 by the tissues exceeds that of T_4 by as much as fivefold, then one would expect to find more T_3 in the tissues of cold-acclimated rats. In fact, this was shown to be the case by two independent studies. Reichlin et al., (1973) showed that the kidney

of the cold-acclimated rat contains more T_3 than the control but no increase was found in the liver or brain; Balsam and Leppo (1974) showed that in the cold-adapted rat there is an increased hormonal binding by the kidney, muscle and liver. Bernal and Rey (1975a and b) showed that the T_3/T_4 ratio of the extrathyroidal tissues of cold-exposed rats increased more than two fold.

Therefore in the rat, cold exposure might increase the conversion of T_4 to T_3 by the extrathyroidal tissues. In fact, this increased conversion might explain why the injection of doses of T_3 lethal to the warm-acclimated rat is tolerated by the cold-acclimated rats (Hsieh, 1962). The cold-acclimated rats might dispose of the hormone at a faster rate.

Part 3: Thyroid hormones - Catecholamines interrelations:

The interrelation between the thyroid gland and the sympathetic nervous system was noticed by scientists from the beginning of modern endocrinology to the present day. There have been many reviews dealing with this interrelation (Harrison, 1964; Leak, 1970; Waldstein, 1966; Gale, 1973). This interrelation will be summarized under the following headings:

a) Clinical observations.

b) Metabolic observations.

c) Mode of interaction.

d) Significanee in cold-acclimation.

Each heading will now be discussed separately:

a) Clinical Observations:

The clinical symptoms of the hyperthyroid state show many similarities to the symptoms of activation of the sympathetic nervous system. It has also been observed that patients with thyrotoxicosis are hypersensitive to catecholamine administration. These observations have led to some clinical applications. For example, adrenergic blocking agents have been used to overcome the symptoms of hyperthyroidism during the time it takes for antithyroid drugs or radioiodine to exert their full therapeutic effects.

b) Metabolic Observations:

The mobilization effects of the catecholamines on the energy reserves are influenced by the thyroid state. In vitro, the lipolytic effect of the catecholamines disappears in the absence of thyroid hormones. On the other hand, the hyperglycemic effect of the catecholamines increases in the presence of excess thyroid hormone and diminishes in hypothyroidism. Also, hyperthyroidism increases and hypothyroidism decreases the calorigenic effect of the catecholamines.

c) Mode of interaction:

Although the actions of the thyroid hormones and the catecholamines are interrelated, the mode of this interaction is still not clear. Two major hypotheses were presented:

i) the catecholamines have a direct effect on the metabolism

of the thyroid gland, and the thyroid hormones have a direct effect on the metabolism of the catecholamines; ii) there exists a factor or a pathway common to both the action of the thyroid hormones and the catecholamines responsible for the mutual interaction.

These two hypotheses were reviewed by Harrison (1964). His conclusion was that there is no substantial evidence for such mutual direct effects (hypothesis i). Hypothesis (ii) is believed to be more probable. The observation that tyrosine is elevated in hyperthyroidism might be a factor since tyrosine is a precursor to both thyroid and catecholamine hormones. The possibility that the two hormones act upon consecutive rate-limiting steps, some authors favouring thyroxine and some catecholamines as acting upon the later step, was considered. Other authors suggested that thyroxine may indirectly decrease the rate at which cyclic-AMP is inactivated, thus potentiating the effects of the catecholamines which are known to stimulate adenylate cyclase resulting in the accumulation of cyclic AMP. If many β-receptor effects are secondary to the accumulation of cyclic AMP, then thyroxine might thus increase the β - receptor activity of the catecholamines. In fact, recently, Rosenqvist (1972) showed that the hypothyroid state decreases the response to noradrenaline in white adipose tissue and smooth muscles by increasing alpha-adrenergic receptor function.

d) Significance in cold-acclimation:

When hypothyroid rats were acclimated to cold, it was found that the increase in the excretion of noradrenaline was more than that in the control cold-acclimated rats (Sellers et al., 1974). The conclusion was that some thyroid hormone is required for cold-acclimation, but that the rats can compensate for mild hypothyroidism by an increased activity of the sympathetic nervous system.

Leblanc and Villemaire (1970) showed that thyroxine and noradrenaline injections into rats gave them a resistance to a cold temperature of -25°C similar to that of cold-adapted rats (at 4°C) transferred to -25° C.

In summary, the thyroid hormones and the catecholamines besides each having unique effects not shared by the other, each can produce independently the effects they share. Therefore, one is considering two independent effectors, the sum of whose effects is greater than their individual contributions. Such a phenomenon is known as synergism (Veldstra, 1956).

Part 4: The calorigenic mechanism of the thyroid hormones:

The biological actions of the thyroid hormone are many and include its calorigenic action, its effects on growth and development, its effects on the turnover of proteins, lipids and other body constituents and its effects on the physiological function of body organs. These effects and others were reviewed by Pitt-Rivers and Tata (1959), and by Barker (1964).

To discuss all these effects of the thyroid hormone is beyond the scope of this thesis. Only the calorigenic effect will be discussed.

Older concepts of the mechanism of the calorigenic effect of the thyroid hormones have emphasized its uncoupling effect on oxidative phosphorylation. This effect, however, is observed only with relatively high doses of hormone (Tata et al., 1963). This uncoupling cannot be accepted, especially since the thyroid hormone stimulates growth and development (and metamorphosis in some forms of life), processes which require large supplies of ATP. It is now generally accepted that liver and muscle mitochondria from hyperthyroid animals have higher respiratory and phosphorylative activity than those from normal controls, but the same ADP/O ratios and respiratory control ratios (Smith et al., 1960, 1962; Degroot et al., 1968; Tata et al., 1963). In fact, recently Sacktor et al., (1972) and Babior et al., (1973) reported that state 3 (ADP-dependent) respiration is more increased than state 4 respiration. They also reported no change in ADP/O ratios or respiratory control.

Therefore, the mechanism of the calorigenic effect of the thyroid hormones must fall under the hypothesis of an increase in energy-requiring processes. The energy-requiring processes believed to be increased by the thyroid hormones are mainly two: i) an increase in the mitochondrial turnover;

ii) an increase in the operation of the sodium pump.

The increase in mitochondrial turnover (i) is evidenced by the results of many workers. Sokoloff and Kaufman (1959) for example, showed that thyroxine administered in vivo or in vitro increased the incorporation of amino acids into the proteins of liver mitochondria, and their conclusion was that this energy-requiring process will result in an increase in the metabolic rate characteristic of hyperthyroidism. In this connection, Booth and Holloszy (1975) showed that the increased content of mitochondrial cytochrome C in liver of thyrotoxic rats appears to be due to an increased synthesis as well as an increased degradation, the former increase being greater than the latter. In addition, Gross (1971) showed that the turnover of both proteins and DNA of the mitochondria of liver and heart increased after thyroxine administration, and decreased after thyroidectomy. On the other hand, Neubert et al., (1970), showed that thyroidectomy results in a decrease in the activities of the nuclear and mitochondrial RNA polymerases, the decrease in the mitochondrial ones being more pronounced. After T_3 administration, the activities of both the nuclear and mitochondrial RNA polymerases increased.

It is known that protein and nucleic acid synthesis requires large amounts of energy. In the case of proteins, the amino acid activation step requires ATP, and during synthesis of the polypeptide on the ribosome, GTP and possibly

other high-energy compounds are required. In addition, large amounts of ATP are required for the synthesis of the nucleotides, the building blocks of the nucleic acids. Thus, this action of the thyroid hormone could contribute to its calorigenic affect.

The effect of the thyroid hormones on the sodium pump has been studied by Ismail-Beigi and Edelman (1970, 1971, 1974). Their studies showed that most of the increase in oxygen consumption after T_4 or T_3 administration could be explained by the increase in the operation of the sodium pump. The measurement of the oxygen consumption due to the sodium pump was done by using ouabain (an inhibitor of the Na-K-ATPase), and then measuring the inhibition of oxygen consumption. The difference between QO_2 in the absence of oubain and QO_2 in its presence was considered to be that amount consumed for the operation of the sodium pump. In addition, the activity of Na-K-ATpase was found to be increased in tissue homogenates after T_3 administration. The tissues that gave positive results were the liver, skeletal muscles and kidney. Cerebral slices and homogenates gave negative results. These results are consistent with the fact that the metabolic rate of the brain is not influenced by the thyroid hormones. The authors also showed that after T_3 administration, the time course of increases in oxygen consumption of the liver in the absence of ouabain, in the ouabain-sensitive respiration, and in the activity of Na-K-ATPase were almost parallel to each other. The conclusion

was that the increased operation of the sodium pump, evidenced by the increase in the Na-K-ATPase and the increased ouabain-sensitive respiration, leads to the increase in oxygen consumption in hyperthyroidism. At this point, it would be interesting to note that the activity of this enzyme was found to be doubled in liver of cold-acclimated rats (Videla et al., 1975).

On the other hand, other authors showed that the thyroid hormone increases the pumping of other ions such as Ca^{2+}. Suko (1971) reported an increase in Ca^{2+} uptake and ATP hydrolysis by the Ca^{2+} -activated ATPase of the sarcoplasmic reticulum in hyperthyroidism, while both were reduced in hypothyroidism. Wallach et al., (1972) reported as increase in the influx of Ca^{2+} and Mg^{2+} by the liver cells after T_4 administration. The authors suggested that these changes in ion transport and intracellular concentration of ions could play a role in the biological actions of the thyroid hormones.

Recent findings give more insight into the mechanism of the calorigenic effect of the thyroid hormone. Babior et al., (1973) showed that the administration of T_3 results in an increase in the mitochondrial uptake of ADP in the liver. They also showed that the increase in ADP uptake could explain the increase in state 3 respiration (ADP-dependent) of the mitochondria. Why would the activity of the adenine nucleotide translocase increase if the rate of ADP uptake is not limiting?

The increased utilization of ATP in the cytoplasm requires that the ADP produced should be rephosphorylated in the mitochondria at a faster rate. It is possible that the translocase, even if working at saturation, cannot cope with the great demand; a synthesis of more enzyme, or an increase in its activity may become essential.

Something which is essential for the operation of the above mechanisms has not been discussed yet. If the cycling of ATP increases in hyperthyroidism, then substrates for oxidation must be delivered at a faster rate. In fact, the thyroid hormones ensure that such a process is accomplished by doing mainly two things: (i) potentiating the mobilization effects of the catecholamines on the body energy reserves; (ii) causing enzyme changes in the cells so that some key pathways are activated.

The first action (i) delivers more substrates to the tissues (see page13). The second action (ii) includes the activation of many enzyme systems in the cell which lead to furnishing substrates for oxidation in the mitochondria at a faster rate. Glock and McLean (1955) reported an increase in the activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in rat liver in hyperthyroidism. The authors concluded that the phosphogluconate pathway for glucose oxidation is more operative in hyperthyroidism. Spiro and Ball (1958), in an attempt to compare the operation of the phosphogluconate

pathway and glycolysis, showed that both pathways are more operative in the hyperthyroid rat.

Lardy et al., (1959) reported a great increase (20-fold) in the activity of the mitochondrial α-gylcerophosphate dehydrogenase in liver after thyroid hormone administration. This increase implied an increase in the operation of the α-glycerophosphate shuttle leading to an increase in glycolysis because of the decrease in the NADH/NAD ratio in the cytoplasm. A thermogenic role was given to this enzyme by Smith (1964) who suggested that the oxidation of α-glycerophosphate is coupled to cytochrome C and therefore only one phosphorylation step occurs on the electron transport chain. Therefore, the oxidation via the α-glycerophosphate pathway proceeds at one third the efficiency of that via the β-hydroxybutyrate pathway in terms of the number of ATP molecules formed, and will give in terms of heat an amount equivalent to two phosphorylation steps. Therefore per one ATP molecule formed, more oxygen would be consumed, more α-glycerophosphate oxidized and more heat produced than when β-hydroxybutyrate is oxidized.

Kubista et al., (1971) also reported an increase in the mitochondrial α-glycerophosphate dehydrogenase in both red skeletal muscles and in heart after thyroid hormone administration. In this connection, Isaacs et al., (1969) showed that the hearts of hyperthyroid rats can utilize lactate in vitro twice as fast as do control rats. The authors ascribed this

finding to the high concentration of NAD in the cytoplasm brought about by the increased operation of the α-glycero-phosphate shuttle.

In general then, the thyroid hormones through their actions at the level of the whole animal, at the organ level and at the cell level, ensure that enough substrate is available to sustain the increased metabolic rate.

The thyroid hormones also seem to increase the capacity of the electron transport system by increasing the levels of some of its components. Drabkin (1950) showed that the tissues of hyperthyroid rats contain increased concentrations of cytochrome C, while the tissues of the hypothyroid rat contain less than one-half the amount found in mormal animals. The author's conclusion was that the thyroid hormones control the tissues' concentration of cytochrome C. Recently, Booth and Holloszy (1975) showed that the increased content of mitochondrial cytochrome C in liver of thyrotoxic rats was due to both an increased degradation and an increased sythesis, the latter exceeding the former. In addition, Beyer et al., (1961) showed that coenzyme Q levels were increased by thyroid injection and decreased by thyroidectomy. At this point, it would be worthwhile noting that these two components, namely, cytochrome C (Klain, 1963) and coenzyme Q (Beyer et al., 1962) levels increase after cold-acclimation.

SECTION 6

ADAPTIVE CHANGES IN MUSCLE FIBERS AND MUSCLE MITOCHONDRIA UNDER PHYSIOLOGICAL, PATHOLOGICAL, AND EXTREME CONDITIONS.

There follows a short review of all those conditions known to produce changes in muscle fibers with emphasis on the changes that occur in the mitochondria. Among such conditions are: exercise, aging, denervation, cross-innervation and reinnervation, disuse atrophy, altitude acclimation, steroid hormones, thyroid hormones and cold-acclimation.

A - Exercise:

The literature is rich in studies on the effects of exercise on muscle fibers and muscle mitochondria. However, there are many conflicting results which make it difficult to draw conclusions about the changes that occur. The confusion exists because the changes that occur after exercise are dependent on:

a) the muscle studied and the type of fibers it contains.
b) the type of exercise (swimming, running, etc.).
c) the duration of exercise each time (10 minutes, one hour, etc).
d) the duration of the exercise period (weeks, months, etc.).
e) the frequency of the exercise (once a day, twice a day, etc).
f) the time at which the studies were made after the exercise.

In view of the confusion in the literature due to these

reasons, it would be useful to summarize those studies which are specific in describing their materials and methods in Table I. This is done in table I. One can see some trends that occur in muscle as a result of exercise. They are the following:

i) The aerobic capacity of skeletal muscles increases after regular physical training. This is believed to be due to the increase in the number and size of the mitochondria as well as the activities of the mitochondrial enzymes. These increases are evidenced by the increased sythesis of total protein, RNA and DNA seen after training in skeletal and heart muscles (Van Linge, 1962; Laguens and Gomez-Dumm, 1968; Rabinowitz and Zak, 1972; Brostrom et al., 1974). The formation of new oxidative enzymes is an adaptive response to the higher demand for ATP by the contractile work. In this connection, Fitts et al., (1975) showed that the endurance of rats (the duration of the run to exhaustion) correlated with gastrocnemius muscle cytochrome C concentration and citrate synthase activity, and with the oxygen uptake capacity of gastrocnemius muscle homogenates.

ii) The muscle shows an increased capacity for prolonged submaximal physical activity. This is believed to occur because of the better ability of muscles after training to keep the lactate level (which causes fatigue)low. The muscle can do that by increasing its capacity to oxidize fatty acids, thus leading to the slowing of glycolysis with a decrease

Table 1: Biochemical effects of exercise on skeletal muscle and muscle mitochondria.

Animal. Muscle. Type of Training Work and duration Reference	Biochemical changes in muscle and muscle mitochondria
Rat. Gastrocnemius Swimming 30 min/day; 8 weeks Hearn and Wainio (1956)	No change in succinic dehydrogenase
Rat. Mixed muscles Swimming 30 min/day; 6 weeks Gould and Rawlinson (1959)	No change in levels of lactic dehydrogenase, malic dehydrogenase and phosphorylase
Rat. Gastrocnemius Running. Several speeds and times 3 months Holloszy, 1967	Increased capacity of isolated mitochondria to oxidize pyruvate. Increase in succinic dehydrogenase, DPNH-dehydrogenase, DPNH-dehydrogenase, DPNH-cytochrome C reductase, succinic oxidase. Increased concentration of cytochrome C and total protein content.
Rat. Gastrocnemius Running 1hr/day; 2 weeks Gollnick and King (1969 a and b)	More numerous mitochondria, larger with more densely packed cristae.

Table 1 (continued)

Rat.Tibialis anterior Swimming 2-3 hr/day; 2 weeks Kraus et al., 1969	Marked increase in mitochondrial size and number, densely packed cristae. Increased capacity of isolated mitochondria to oxidize pyruvate. Increase in glycerol phosphate dehydrogenase, succinic dehydrogenase. Increase in cytochromes content and mitochondrial protein. Phosphorylation coupled. No change in glycolytic enzymes.
Rat. Gastrocnemius Running, several speeds and times 3 months Holloszy et al., (1969) (1970)	Increase in enzymes of citric acid cycle Decrease in mitochondrial α-glycerophosphate dehydrogenase.
Rat. Gastrocnemius, Quadriceps, Soleus 2 hr/day; 12 weeks Baldwin et al., (1972)	Increased capacity to oxidize pyruvate and palmitate. Increase in levels of cytochrome oxidase, carnitine palmityltransferase and citrate synthase. Increase in cytochrome C.
Rat. Gastrocnemius, Quadriceps, Soleus Running 2 hr/day; 10 weeks Askew et al., (1973)	Increase in glycerol-3-phosphate esterification (27%). Therefore training results in an increase in a synthetic pathway of lipid metabolism.
Rat.Rectus femoris soleus Short term-high intensity (sprint) training Staudte et al., (1973)	Increase in hexokinase, glycogen phosphorylase, triosephosphate dehydrogenase, citrate synthetase, and succinate dehydrogenase. No increase in fatty acid oxidation (no increase in 3-hydroxyacyl-CoA-dehydrogenase.)
Rat. Gastrocnemius, Quadriceps Running, 2hr/day 12 weeks Molé et al., (1971)	Increase in the levels of palmityl CoA synthase, carnitine palmityl transferase and palmityl CoA dehydrogenase, and in the capacity to oxidize fatty acids. Increase in mitochondrial protein.

Table 1 (continued)

Rat. Gastrocnemius Running 1 hr/day; 10 weeks Gollnick et al., (1971)	Increase in number and size of mitochondria with densely packed cristae. Increase in succinic dehydrogenase and mitochondrial proein.
Rat. Gastrocnemius Treadmill running 12 weeks Holloszy et al., (1973)	Muscle homogenates oxidize D-β-hydroxybutyrate-3-14C 2 to 3 times as rapidly as did homogenates of muscles from sedentary animals. This might explain why physically trained individuals have greater resistance to ketosis than the untrained during and after prolonged exercise.
Rat. Gastrocnemius 8 hours of treadmill running Kendrick-Jones and Perry (1965)	An increase in aldolase activity.
Rat. Gastrocnemius 30-40 minutes of Swimming with a load. Brostrom et al., (1974)	No changes in pyruvate kinase activity. An increase in the RNA content. An increase in phosphofructokinase activity. An increase in the activities of the hexose monophosphate dehydrogenases.

in the rate of formation of pyruvate and extramitochondrial NADH. In this connection, Fitts et al., (1975) showed that there was an inverse relationship between the amount of glycogen depleted from liver and muscle during exercise and the respiratory capacity of the rat's leg muscles.

iii) The skeletal muscles after training become capable of taking up ketone bodies and oxidizing them more rapidly (Holloszy et al., 1973). This is why physically trained animals have a greater resistance to ketosis. This change might be a reflection of the increase in the amount of β-hydroxybutyrate dehydrogenase enzyme in skeletal muscle mitochondria.

iv) Some authors believe that with exercise, there is a shift in fiber population towards the red oxidative fibers (Syrovy et al., 1972), thus resulting in an increase in the amounts of oxidative enzymes.

B - Aging:

It is known that the skeletal muscles decrease in weight with age if there is no training. This decrease in weight was shown to be the result of both: i) a decrease in the number of fibers especially the red ones; ii) a decrease in the volume of the fibers (Faulkner et al., 1971; Tauchi; et al., 1971;Lieberman et al., 1972).

In their comparative studies on the heart structure of young and old rats, Tomanek and Karlson (1973) showed that in the hearts of the old rats there were aggregations of dense

mitochondria. The authors suggested that these aggregations were caused by the proliferation of the mitochondria in response to the decrease in the muscle's capacity to utilize oxygen.

In addition, comparative studies were made on the respiratory and phosphorylative properties of heart and skeletal muscle mitochondria of young and old rats. Chen et al., (1972) found no change in the ADP/O ratios nor in the state 4 respiration (in the absence of ADP) with all substrates. However, there was a decrease in state 3 respiration (ADP-dependent) with some substrates. The authors suggested that aging brings with it a change in the coupling mechanism leading to some loss in the respiratory control with some substrates.

C - Denervation:

When a muscle is denervated, its contact with the nervous system is abolished, and biochemical changes are expected to occur leading in the end to atrophy. After denervation, all types of muscle fibers show a gradual decrease in the activities of the enzymes of energy metabolism. The fast, glycolytic (white) fibers lose more rapidly their glycolytic enzymes, and the slow, oxidative (red) fibers lose their oxidative enzymes, so that the metabolic differences between the two types of fibers disappear (Romanul and Hogan, 1965; Hogan et al., 1964). These changes are then followed by decreased respiratory activity of the mitochondria, and decreased activity of the mitochondrial enzymes, thus leading

to the eventual atrophy (Romanul and Hogan, 1965).

Mitochondrial changes in shape, size and distribution were also reported 24 hours after denervation (Slater and Miledi, 1968); and fragmentation of the fiber mitochondria was reported by Muscatello and Patriarca (1968). In fact, these changes are expected since the muscle is on the way to atrophy.

The interesting finding here is that after denervation, the muscle fibers undergo metabolic differentiation to a homogeneous type of new fibers. This finding raises the possibility that the energy metabolism of the muscle fibers is determined by their nerve supply. In fact, this was proven in cross-innervation and reinnervation experiments.

D - Cross-innervation and reinnervation:

Romanul and Van Der Meulen (1966, 1967) carried out experiments on young and adult rats and cats in which the nerves to the soleus muscle (slow) were sectioned and cross-united in some animals to the fast flexor digitorum longus (FDL) or flexor hallucis longus (FHL) which were also sectioned; in other animals, reunited the sectioned nerve of the soleus back to the soleus. Then, they performed the opposite operation in which the nerves of the fast muscles (FDL or FHL) were sectioned, cross-united or reunited. Four to ten months after these operations, the cross innervation experiments showed that the soleus has changed to become a fast muscle rich with

glycolytic fibers, and that FDL and FHL have changed to become muscles characteristic of the slow oxidative type. On the other hand, the reinnervation experiments resulted in no change in the fiber type of the muscles. The involvement of the mitochondria in the above differentiations is clear, since the oxidative enzymes are mitochondrial. The conclusion drawn from these studies was that the preferential energy metabolism of the muscle fibers is determined by their nerve supply. In fact, there is a correlation between the metabolism of the muscle fibers, their speed of contraction and the physiological characteristics of the nerve fibers that innervate them (Eccles et al., 1958). In this connection, Pette et al., (1973) showed that the intermittent long-term stimulation of fast rabbit muscles with a frequency pattern resembling that of a slow muscle led to the transformation of the fast muscle to a slow muscle as evidenced by histochemical staining, and rearrangement of the enzyme activities, resulting in a decrease in the activities of the enzymes of glycogenolysis and glycolysis and an increase in the activities of the enzymes of the citric acid cycle, of fatty acid activation and oxidation, and of ketone body utilization.

E - Disuse atrophy:

Disuse atrophy of the muscles of the hind limb can be produced by driving a needle through the calcaneous into the shaft of the distal tibia, and another needle through the

distal femur into the proximal tibia. In this way, the limb is immobilized and disuse atrophy will result. Max et al., (1973) performed this kind of immobilization and studied the response of the mitochondrial enzymes of the gastrocnemius muscle to the decreased activity. The authors found that the mitochondrial yield from the atrophic muscles decreased gradually. They also found that the activities of cytochrome oxidase and monoamine oxidase per total muscle homogenate decreased, but the specific activities in isolated mitochondria did not change. The authors concluded that there are fewer mitochondria. In addition, the specific activity of malate dehydrogenase in the isolated mitochondria was found to decrease up to 35% of the control by the 15th day. The authors suggested that the decrease in the activity of this matrix enzyme is a reflection of different rates of turnover of mitochondrial components, and not a reflection of permeability changes since the nicotinamide nucleotide, the Ca^{2+} levels and NADH oxidation were not different from the control.

In addition, Max (1972) showed that the mitochondria under these conditions had very low ADP/O ratios and respiratory control which appeared as early as the first day after immobilization and progressed until the 6th day at which time, the ADP/O ratio could no longer be measured, and the respiratory control was completely lost.

F - Altitude-acclimation and hypoxia:

Significant changes occur in the mitochondria of mammals acclimated to hypobaric pressures. Shertzer and Cascarano (1972) studied the mitochondrial changes in heart muscle and in other tissues of rats maintained at 0.5 atmosphere for 14 days followed by 14 days at 0.4 atmospheres. The hypoxia-acclimated animals showed a 26% decrease in cytochrome oxidase activity per gram wet weight, but no change when the activity of the enzyme was expressed per unit of mitochondrial protein. The authors concluded that the mitochondrial mass is diminished in heart muscle after altitude acclimation. In other work, Rabinowitz et al., (1971) exposed rats to 6-hour periods of 4-5% oxygen and found that this kind of hypoxia led to the destruction of the mitochondria. However, when the animals were returned to normal atmosphere, rapid synthesis of mitochondrial proteins occured.

The work of Shertzer and Cascarano (1972) also showed that after the altitude acclimation, the activity of succinic dehydrogenase per unit mitochondrial protein increased by 41%. In addition, the concentrations of cytochrome a + a_3 per unit of mitochondrial protein showed little change, while cytochromes b and c + c_1, as well as succinate-reduced flavoprotein increased significantly.

G - Steroid hormones:

As far as I know, no study of enzymic changes has been

done on muscle and muscle mitochondria after glucocorticoid administration. Some microscopic studies were done showing that more enlarged mitochondria appear especially next to the subsarcolemmal zone. In addition, some destruction of the mitochondria appeared in deeper zones, in the interfibrillar regions. These changes were observed in both red and white fibers (Engel, 1967; Bullock et al., 1971). In a later study, Bullock et al., (1972) showed that some of the corticosteroids caused a significant fall in the respiratory control ratio of the mitochondria with some substrates after 12 hours administration.

H - Thyroid hormones:

Zaimis et al., (1969), reported an increase in the size and number of mitochondria in the hearts of hyperthyroid cats, but only an increase in size in the guinea pig. The authors also reported the existence of mitochondrial pleomorphism, and decreased mitochondrial matrix in both hyperthyroid cats and guinea pigs. Recently, Callas and Hayez (1973) showed that the administration of thyroid hormones resulted in changes in heart mitochondria. The mitochondria became larger in size but did not increase in number. The cristae were disoriented and there were localized areas of vacuolization. The authors also showed that these changes were reversible since a return to the euthyroid state was accompanied by the disappearance of these changes. The authors suggested that since the changes are

reversible, then they must reflect the increase in the metabolic activity brought about by the hyperthyroid state.

The mitochondria of skeletal muscles were also studied in thyroxine-treated rats. Gustafsson et al., (1965) reported an increase in size, and a 3-fold increase in the total number of the mitochondria. However, in this case, these mitochondrial changes were found to persist after 3 weeks of cessation of hormone administration even though the oxygen consumption had returned to normal at that time. This implied that the mitochondrial structural changes were not correlated with the increase in the metabolic rate. It was possible however, that the necessary changes that could bring back the basal metabolic rate to normal have occured even though not observed by electron microscopy. If Gustafsson had waited a longer time, he might have observed normal mitochondria again. By the same token, the increase in oxygen consumption after hormone administration could have preceded the observed structural changes in the mitochondria.

On the other hand, Winder et al., (1975) observed no change in the skeletal muscle mitochondria of rats after thyroid hormone administration, even though the authors used the same doses that produced changes when used by other authors.

These conflicting results call for caution in interpreting electron microscopy studies. Thus, the exact effects of the thyroid hormones on muscle mitochondria remain to be settled.

As to the functional and enzymic changes in muscle mitochondria caused by the thyroid hormones, these were discussed in section 5 under the mechanism of the calorigenic action of the thyroid hormones.

I - Cold-acclimation:

It is believed that prolonged cold exposure leads to increases in the activity of certain enzymes and cofactors associated with electron transport system of skeletal muscles of the rat. Hannon (1960) reported increases in the activities of succinate dehydrogenase and cytochrome oxidase; Beyer et al., (1962) reported increased levels of coenzyme Q (ubiquinone); Klain (1963) reported increased levels of cytochrome C. Depocas (1966) showed that while the average concentration of cytochrome C in all skeletal muscles was higher in cold-acclimated rats than in warm-acclimated rats, yet the concentration of cytchrome C did not increase in all the skeletal muscles tested so that the total amount of skeletal muscle cytochrome C per rat did not change. However, there are some conflicting results in the literature. For example, Hamilton and Ferguson (1972) found no change in the activity of succinate dehydrogenase; Himms-Hagen et al., (1975) found no change in the activity of cytochrome oxidase.

As to structural changes, Behrens and Himms-Hagen (in press) showed that the mitochondria of both red and white skeletal muscles of cold-acclimated rats become increased in number but decreased in size.

In conclusion, the skeletal muscle mitochondria are flexible in the sense that they can adapt to external and internal conditions. However, it is clear from the above discussion that the studies made on such adaptations are not consistent and sometimes confusing, which makes it very difficult to draw conclusions.

Statement of the problem

Nonshivering Thermogenesis (NST) is an adaptation in which there is a high metabolic rate that can be switched on and off according to the needs of the animal. The mechanism of NST is still not understood, but since the skeletal muscles are believed to be the major site of NST (see page 5), then one might expect to find changes in the mitochondria of this tissue, especially since the flexibility of mitochondria to environmental changes is well documented. (see page 40). In fact, recently, ~~Behrens~~ and Himms-Hagen (in press) showed that the skeletal muscle mitochondria of cold-acclimated rats become smaller in size but more numerous.

Thus, the aim of this work was to look for specific biochemical changes in the skeletal muscle mitochondria of cold-acclimated rats that might shed some light on the mechanism of NST in this tissue. The comparative characteristics studied were the following:

1 - The respiratory properties of the isolated skeletal muscle mitochondria of both warm and cold-acclimated rats.

2 - The levels of some enzymes of the Krebs cycle, and related enzymes.

3 - Two major types of mitochondrial-cytosolic interactions namely, the transport of reducing equivalents, and the transport of ADP from cytosol into mitochondria.

CHAPTER B

MATERIALS AND METHODS

1 - Materials:

a) Rats:

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

b) Chemicals:

ATP (Adenosine-5'-triphosphate, disodium salt), ADP (adenosine-5'-diphosphate, sodium salt), Tris (tris-hydroxymethyl-aminomethane), EDTA (ethylene diamino tetraacetic acid, tetrasodium salt), Rotenone (Grade II), sucrose, mannitol, heparin (117 units/mg), protease (subtilisin BPN'; 6.6 units/mg), HEPES (N-2-hydroxyethylpiperazine -N'-2 ethanesulfonic acid) buffer, bovine serum albumin (low fatty acid content), DL-α-glycerophosphate

(disodium salt, grade X), succinic acid, α-ketoglutaric acid (disodium salt), pyruvic acid (sodium salt), malic acid (sodium salt), malonic acid (sodium salt), glutamic acid, phosphoenolpyruvate (trisodium salt), isocitric acid (sodium salt), β-hydroxybutyrate (sodium salt), PMS (phenazine methosulphate), INT (iodonitrotetrazolium violet, grade 1), β-NAD (β-nicotinamide adenine dinucleotide, grade III), β-NADH (β-dihydronicotinamide adenine dinucleotide phosphate, monosodium salt), atractyloside (potassium salt), lactic dehydrogenase (LDH-5(M_4) isoenzyme, from rabbit muscle, type V), malic dehydrogenase (from pig heart), pyruvate kinase (from rabbit skeletal muscle, type II), myokinase from rabbit muscle, (grade III), glucose-6-phosphate dehydrogenase (from Torula Yeast, type XI), phosphoglucose isomerase (from yeast, grade III), were purchased from Sigma Chemical Company. Potassium cyanide (KCN), trichloracetic acid (TCA) and ethyl acetate were purchased from Fisher Scientific Company. Potassium phosphate (monobasic and dibasic) were purchased from J.T. Baker Chemical Company. (U-^{14}C) Adenosine 5'-diphosphate (ammonium salt) was purchased from Amersham/Searle Corporation.

For the electron microscopy studies, the following chemicals were used:

Glutaraldehyde: Ladd Reseach Industries, Inc., 70% glutaraldehyde, cacodylate acid; sodium cacodylate: Sigma

Chemical Co., osmium tetroxide: Electron Microscopy Sicences, ethanol: Liquor Control Board of Ontario, styrene: Eastman Kodak Co., vestopal-W, initiator and activator and resin: Mme Martin Jaeger, Geneva., lead citrate: Electron Microscopy Sciences, uranyl acetate: British Drug Houses, Analar.

2 - Methods:

a) Removal of the skeletal muscles:

The rats were killed by decapitation at room temperature after determination of their body weight. The muscles of the hind legs were then quickly exposed, and removed with scissors and transferred to a cold 0.15M KCl solution. The muscles were then cleaned by removing the fat and connective tissues as much as possible. This was done as fast as possible and on a cold plastic plate. The muscles were then minced very quickly on a wooden plate using a very sharp razor blade, and then weighed. Usually, 10 grams of muscle mince was used for further operations.

b) Preparation of the mitochondria:

The method described here was worked out by Behrens and Himms-Hagen (unpublished work). The entire operation was carried out between 0 and 4° C. The solutions used were:

a Mannitol isolation medium (M.I.M.)	b Mannitol isolation +medium Heparin (M.I.M + H)	c Digestion medium (D.M.)
Mannitol 0.21 M Sucrose 0.07 M EDTA 0.01 M HEPES buffer 0.01 M pH = 7.4	M.I.M. + Heparin 250 Units/ml pH = 7.4	M.I.M.+H(100ml.) ATP (1 mM) 20 mg protease pH = 7.4

i - The digestion medium was prepared freshly before the experiment. The muscle mince was added to 100 ml. of the freshly prepared digestion medium and stirred for 30 minutes on ice at about 120 r.p.m.

ii - After the 30-minute digestion period, the contents were homogenized (motor driven) in a loosely fitting all-glass Potter-Elvehjem homogenizer for 10 seconds. The homogenate was then submitted to differential centrifugation as follows:

iii - The muscle homogenate was poured into 4 X 50 ml. plastic centrifuge tubes and centrifuged at 650 x g for 5 minutes in a swinging bucket rotor (HB_4) in a Sorvall Superspeed RC 2-B centrifuge.

iv - The supernatant was filtered through cheesecloth into 4 x 50 ml. tubes and the sediment discarded. The supernatants were topped with M.I.M. + H medium and centrifuged at 13,000 x g for 15 minutes.

v - The supernatant was discarded. The pellet was suspended in M.I.M. (without heparin) and homogenized smoothly by hand

in a glass homogenizer with a teflon pestle. The suspended mitochondrial pellet was then centrifuged at 10,000 x g for 10 minutes.

vi - The supernatant was discarded, and the mitochondrial pellet was resuspended in the M.I.M. (without heparin) and homogenized as in part v. The homogenate was then centrifuged at 650 x g for 5 minutes.

vii - The supernatant was saved, and the sediment was re-suspended in the M.I.M. (without heparin) and homogenized as in part v. The homogenate was then centrifuged at 650 x g for 5 minutes.

viii - The supernatant was added to the saved supernatant of part vii and the pellet was discarded. The two supernatants combined make up the mitochondrial suspension from which samples were taken for protein estimation and electron microscopy studies.

ix - The mitochondria were finally centrifuged at 10,000 x g for 10 minutes. The supernatant was discarded. The pellet was then resuspended in different solutions depending upon the experiments to be performed. These will be described in the results section under: "description of the experiment".

c) <u>Fractionation of the mitochondria into membrane and matrix fractions</u>.

i - The final mitochondrial pellet was suspended in 0.2 M phosphate (KH_2PO_4) buffer, pH 7.5 at a concentration of 1 mg/ml, by hand, using a glass homogenizer with a teflon pestle.

ii - The suspension was sonicated for 2 minutes at high power output, but interrupted after every 20-second interval for cooling. The sonicator used was the Biosonik III (Bronwill Scientific), and the intensity setting was at maximum using the large size probe. The suspension was kept close to 0°C on salted ice.

iii - The sonified suspension was centrifuged at 100,000 x g for one hour. The supernatant was saved. The pellet was resuspended in the phosphate buffer as in part i and centrifuged again at 100,000 x g for one hour. The supernatant was then added to the first one to make up the matrix fraction. The pellet was finally suspended in phosphate buffer as in part i. This suspension makes up the membranes fraction (Klingenberg, 1967).

d) Preparation of the cytoplasmic fraction of the muscle fibers:

The muscles were removed and cleaned as described before. The muscles were then homogenized in a mortar and pestle in the presence of neutral alumina oxide (Lee and Lardy, 1965).

The solution used for homogenization varied depending upon the enzymes to be assayed. When the cytoplasmic α- glycerophosphate and malate dehydrogenases were being studied, the solution was 0.25 M sucrose. In this case, 2 grams of cleaned muscle were homogenized in 6 ml. of 0.25 M sucrose (Lee and Lardy, 1965). When the cytoplasmic alanine and aspartate transaminases were being assayed, the solution

used was 0.25 M sucrose - 1mM EDTA, pH = 7.0. In this case 2 grams of cleaned muscle were homogenized in 8 ml. of 0.25 M - 1mM EDTA, pH = 7.4 (Gatehouse et al., 1967).

The homogenates were then centrifuged at 100,000 x g for one hour. The supernatant was used for the enzyme assays (Lee and Lardy, 1965).

e) Protein estimation

The method used for protein estimation was a modified Lowry et al., (1951) method developed by Schacterle and Pollack (1973). However, due to the interference of HEPES buffer with the method, the following preliminary steps had to be carried out:

Duplicates of 0.1, 0.2 and 0.3 ml. of the sample were added to 6 centrifuge tubes. Then 2 ml. of 12.5% TCA was added to each aliquot, and allowed to stand on ice for 30 minutes, after which they were centrifuged at 10,000 x g for 5 minutes. The supernatants were discarded, and the pellets (proteins), each disolved in 1 ml. of 0.5 N NaOH.

The following steps were those of Schacterle and Pollack (1973).

f) Mitochondrial respiration

The set-up used for the study of mitochondrial respiration was the YSI oxygen monitor, model 53. It consisted

of the following:

a - The oxygen meter, Model 53 (Yellow Springs).

b - The bath, stirrer assembly.

c - The oxygen sensor (a Clark polarographic sensor).

d - A pump, thermostated, circulating, Haake, Model FE.

e - A Beckman recorder (Model 1005) was connected to the oxygen meter.

The measurement of the mitochondrial respiration is described in "description of the experiment" part of the results section.

g) Enzyme assays:

The assay procedures for all the enzymes studied in this work are described under the "description of the experiment" part of the results section.

h) Electron Microscopy:

Mitochondria were fixed in suspension by adding 2.5 ml of ice cold 6% glutaraldehyde in 0.02 M sodium cacodylate buffer, pH 7.0 to 5 ml of mitochondrial suspension containing 5 mg of protein (Munn and Blair, 1967). After one hour the suspension was centrifuged for 30 minutes at 10,000 rpm (25,000 x g) and the supernatant was replaced by 3 ml of 1% osmium tetroxide in 0.02 M sodium cacodylate buffer pH 7.0 The osmium tetroxide solution was replaced once after a further 30 minutes of centrifugation. After another 30 minutes of centrifugation the osmium tetroxide solution was replaced, the pellet broken into small fragments with a finely drawn

glass rod and the fragments allowed to remain in the osmium tetroxide for another hour. The fragments were dehydrated successively in 50%, 75%, 95% and 100% ethanol, transferred to styrene and then embedded in Vestopal-W. Impregnation with Vestopal was allowed to proceed for 2 days at room temperature as recommended by Nunn (1970) before polymerization at 60° for 4 days. Thin sections (silver) were cut with a Reichert Ultramicrotome (UM-2) using glass knives. Sections were stained with lead citrate (Venable and Coggeshall, 1965) for 4 minutes and with uranyl acetate (saturated solution in 60% ethanol) for 4 minutes. Specimens were viewed in a Siemens 101 electron microscope at 7000 x magnification.

i) Statistical analysis of the results:

The statistical analysis of the results were performed according to Snedecor (1965). The results represent means $\pm$ standard errors and the significance was measured by a student "t" test.

CHAPTER C

RESULTS

SECTION 1

DETERMINATION OF THE PURITY OF THE ISOLATED MITOCHONDRIA OF THE SKELETAL MUSCLES OF COLD-AND WARM-ACCLIMATED RATS BY ELECTRON MICROSCOPY

i - Purpose of the Experiment:

It is necessary in every piece of work directed towards the understanding of mitochondria, but performed on mitochondrial fractions to raise and, if possible, to answer the following questions:

a) What is the likelihood that the results obtained express properties of contaminants rather than of the mitochondria themselves ?

b) If the observed properties are truly mitochondrial, what is the likelihood that they have been modified or affected in any way by the presence of contaminants ?

Such questions are not easily answered, but this is no reason to ignore them. It is the purpose of this section to determine by electron microscopy the purity of the mitochondrial fractions.

ii - Description of the experiment:

The method used for the electron microscopy has been described in chapter B, part 2 (h).

iii) - Results and discussion:

The electron micrographs of the mitochondria of the skeletal muscles (red and white) of cold- and-warm-acclimated rats are shown in figures 1 and 2 respectively. The mitochondria are recognized morphologically by the presence of two membranes, of which the inner one shows infoldings or cristae.

The mitochondria in both show a variety in shape, size and in conformation. It is obvious in both figures that the mitochondrial fractions are almost entirely mitochondria, however there are some contaminants, although a minor fraction. These contaminants might be nuclear fragments, cytoplasmic granules, cell membrane components, fibrous material, or any combination of these.

A comparison of figures 1 and 2 shows the following:

a) In figure 2, the section is thicker and this explains why the background looks darker and more electron dense, leading to the exaggeration of the differences between figures 1 and 2.

b) The mitochondria in both figures show the typical three types of configurations of isolated muscle mitochondria described by Weber (1972). They are the following:

i - The coalesced and aggregated configuration in which the inner membranes "come together" in many of the inmerices (the structures composed of two inner membranes plus enclosed matrix), giving them a honeycomb appearance in many instances.

Figure 1: Electron micrograph of the isolated skeletal muscle mitochondria of a warm-acclimated rat at 7000 x magnification.

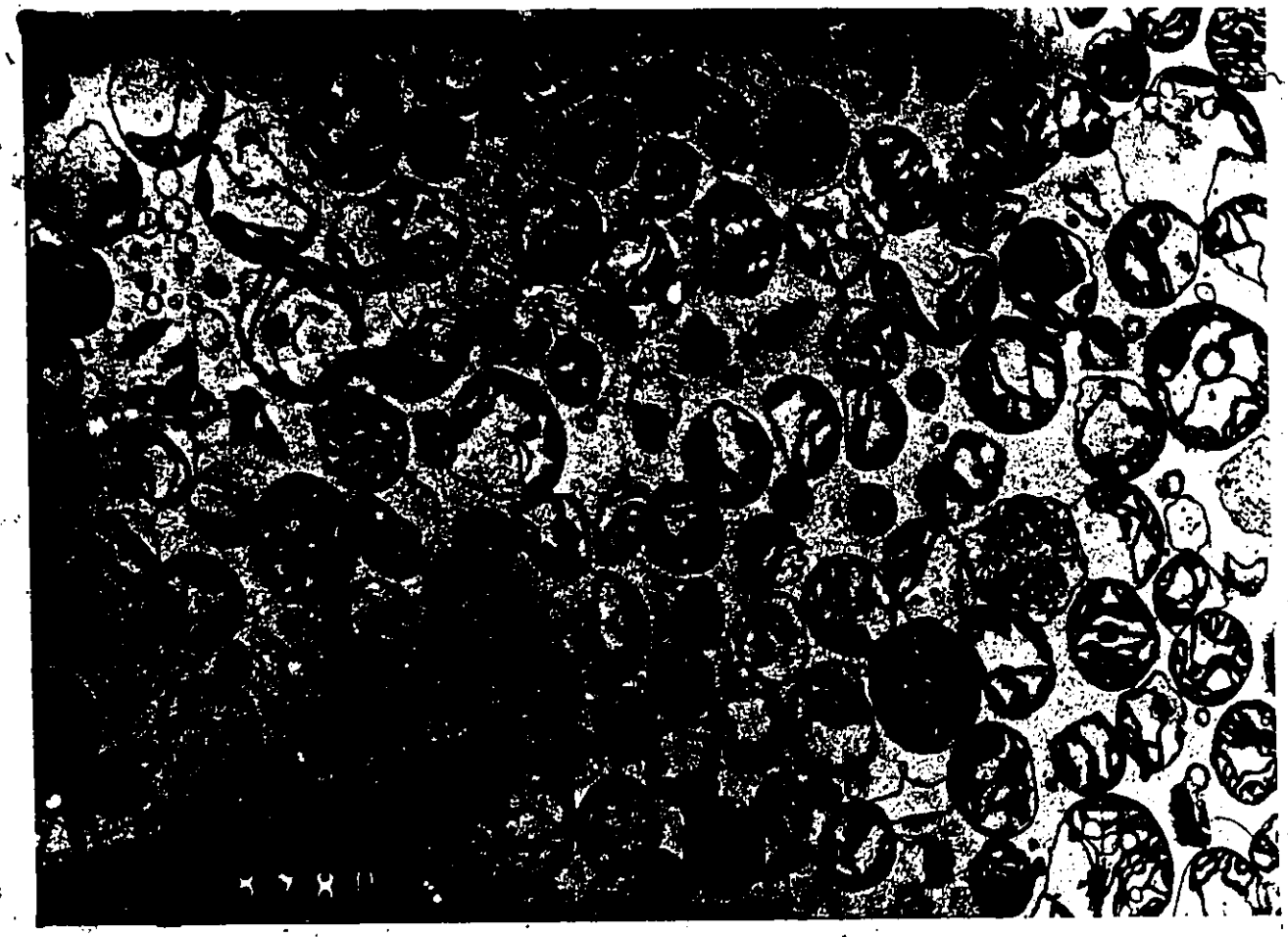


Figure 2: Electron micrograph of the isolated skeletal muscle mitochondria of a cold-acclimated rat at 7000 x magnification.

This configuration is predominant in figure 1 and this might be the reason why these mitochondria look less intact. In fact this is the appearance characteristic of isolated skeletal muscle mitochondria from normal rats (Kuner and Beyer, 1970).

ii - The distended configuration in which the inmerices have a twisted tubular appearance at many points, and areas that appear distended (swollen) in many inmerices are almost circular in most cases. This configuration is predominant in figure 2.

iii - The condensed configuration in which the majority of the swollen and twisted inmerixal areas of the distended configuration have disappeared or condensed. This configuraton is predominant in figure 2.

Thus, the apparent difference between mitochondria from a warm-acclimated rat (figure 1) and mitochondria from a cold-acclimated rat (figure 2) would appear to be due to a difference in metabolic state (Weber, 1972). The reason for this difference is unknown. It is unlikely to be due to a difference in coupling since these mitochondria were found to be tightly coupled (see section 2 in chaper C).

It appears therefore that the mitochondrial fractions isolated in this work are almost entirely mitochondria. This fact is essential for later work.

SECTION 2

DETERMINATION OF THE RESPIRATORY RATE, THE ADP/O RATIO, AND THE RESPIRATORY CONTROL OF THE SKELETAL MUSCLE MITOCHONDRIA OF COLD AND WARM-ADAPTED RATS.

i) Purpose of the experiment: to explore the respiratory and oxidative properties of the isolated mitochondria of muscle of cold-acclimated rats, and to compare them with those of the warm-acclimated rats. The possibility was that the former mitochondria might be loosely coupled.

ii) Description of the experiment:

The mitochondria from both warm and cold-acclimated rats were prepared as mentioned in the methods. They were suspended in a medium composed of 0.021 M mannitol, 0.07 M sucrose, 0.01 HEPES buffer pH 7.4, 0.01 M EDTA, heparin 250 units/ml and 1% bovine serum albumin (low fatty acid content).

Respiration was measured polarographically at 37°C in a medium containing 0.015 M potassium chloride, 0.025 M Tris buffer pH 7.4, 0.007 M EDTA, 0.045 M sucrose, 0.005 M magnesium chloride and mitochondrial protein, 0.5 mg/ml (Max et al.,1972).

Respiration was measured in the presence of one of the following substrates: 5 mM α-glycerophosphate, 5mM succinate (FAD-linked substrates), 10 mM α-ketoglutarate, 10 mM pyruvate + 10 mM malate, 10 mM α-ketoglutarate +10mM malonate (NAD-linked substrates).

State 3 respiration was measured in the presence of 0.2

mM ADP in the case of the FAD-linked substrates, or 0.4 mM ADP in the case of the NAD-linked substrates. State 4 respiration is the rate after stimulation by ADP has ceased.

The steps of the measurements were as follows:

a) The incubation medium was added first and allowed to equilibrate with the 37°C temperature for 5 minutes.

b) The mitochondria were added and allowed to incubate for 3 minutes. The total volume was 3 ml.

c) The substrate was finally added after the tracing on the chart was switched on. A low rate of respiration was initiated.

d) After 2 minutes of the above low rate of respiration 10 µl. of $K_2 HPO_4$ (pH=7.4) was added at a concentration of 10 mM. A rapid increase in the rate of respiration occurred. This condition was allowed to proceed for another 2 minutes.

e) ADP was then added, and a rapid increase in the rate of respiration started. This condition is known as state 3 respiration. It was allowed to proceed until all the ADP had been phosphorylated, after which the rate returned to a value approximately equal to that after the Pi addition. This last rate of respiration is known as state 4 respiration. The volume of ADP solution added was 10µl.

f) The rates of respiration in all the above conditions were calculated by assuming that at 37°C the solubility of oxygen in the incubation medium is 1.2 microatoms/3ml.

g) The ADP/O ratios were obtained by dividing the number of micromoles of ADP consumed by the number of microatoms of oxygen used for this consumption.

h) The respiratory control ratio was obtained by dividing the rate of state 3 respiration by that of state 4.

i) As a specific example of all the above calculations, a typical tracing is presented in figure 3.

iii) Results and discussion:

The results of the experiment are shown in table II and figure 4. As can be seen in the table, no changes were found in the ADP/O ratios of all substrates studied. The respiratory control ratios did not change also for all substrates except for α-glycerophosphate where the respiratory control ratio increased. The results also showed that the mitochondria from the cold-acclimated rat have greater respiratory and phosphorylative activities as evidenced by the increases in state 3 respiration for all the substrates studied and increases in state 4 respiration for most of the substrates studied (no changes for glutamate or α-ketoglutarate + malonate).

From table III, the increases in the rates of respiration in states 3 and 4 for all the substrates can be seen more clearly. First, one should explain the increase in the state 4 respiration of the FAD-linked substrates and some of the NAD-linked substrates (α-ketoglutarate and pyruvate + malate). It is possible in this case that there are increases in the activities of the mitochondrial dehydrogenases for these substrates or increases in the permeability to the substrates . Table III also shows that the absolute increases in state 4

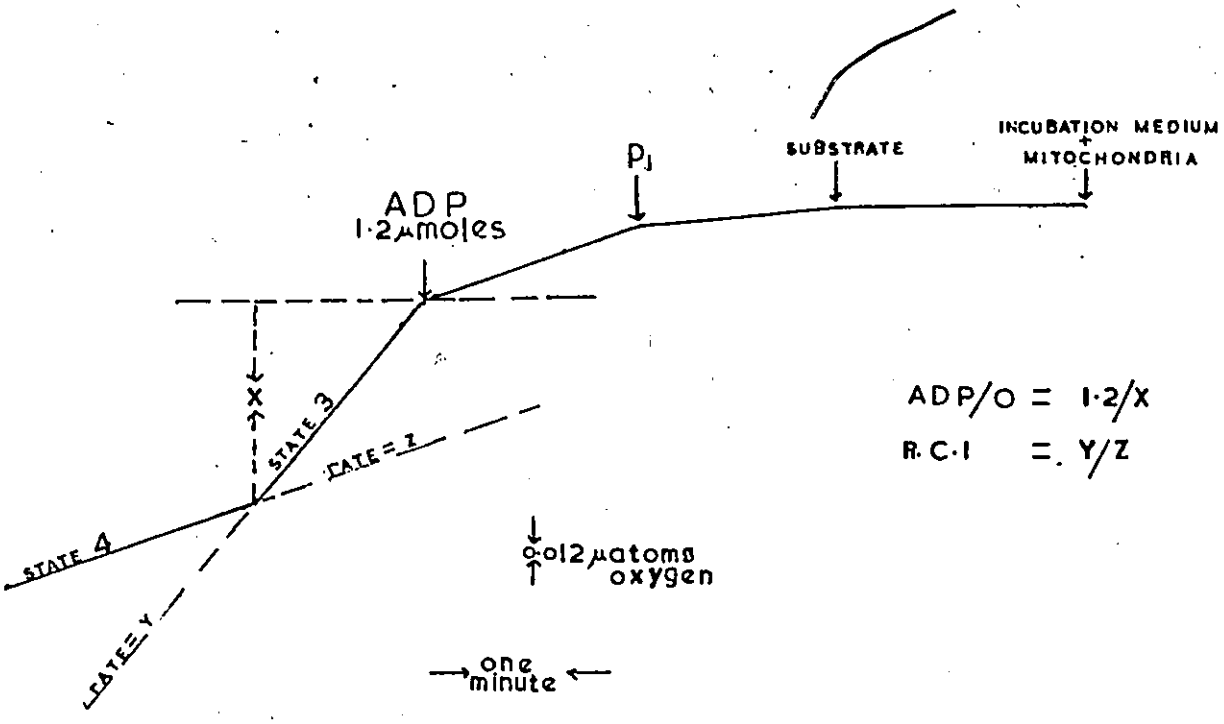


Figure 3. Typical tracing of phosphorylating mitochondria.

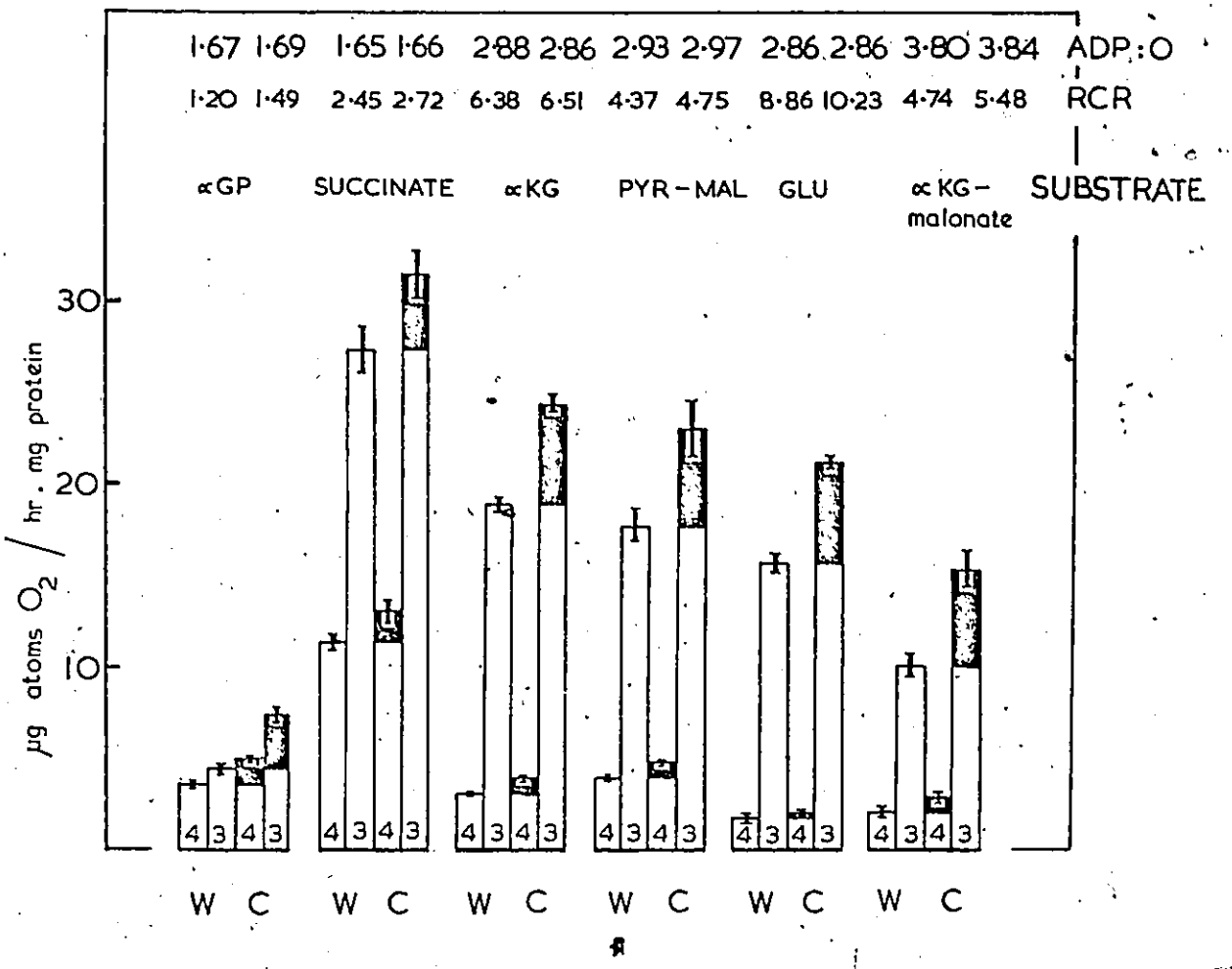


Figure 4. Respiratory and oxidative properties of skeletal muscle mitochondria of cold- and warm-acclimated rats.

Table II. Respiratory and oxidative properties of muscle mitochondria of warm and cold-acclimated rats.

Substrate	Warm				Cold			
	μ g atoms O /hr/mg		ADP/O (μmoles ADP / μ g atoms oxygen)	R.C.R. (State 3/ State 4)	μ g atoms O/hr/mg		ADP/O (μmoles ADP / μ g atoms oxygen)	R.C.R (State 3/ State 4)
	State 4	State 3			State 4	State 3		
α-glycero-phosphate n = 9	3.66 ±0.24	4.40 ±0.3	1.67 ±0.039	1.2 ±0.024	4.9 ±0.16 P<0.001	7.39 ±0.32 P<0.001	1.69 ±0.027 NS	1.49 ±0.048 P<0.001
Succinate (Rotenone) n = 9	11.5 ±0.40	27.66 ±1.21	1.65 ±0.036	2.45 ±0.112	13.13 ±0.58 P<0.05	31.51 ±1.32 P<0.05	1.66 ±0.042 NS	2.72 ±0.132 NS
α-ketoglu-tarate n = 4	3.05 ±0.064	18.98 ±0.37	2.88 ±0.03	6.38 ±0.236	3.80 ±0.079 P<0.001	24.70 ±0.51 P<0.001	2.86 ±0.031 NS	6.51 ±0.246 NS
Pyruvate + malate n = 4	4.22 ±0.134	17.86 ±0.958	2.93 ±0.027	4.39 ±0.25	4.92 ±0.079 P<0.005	23.33 ±1.48 P<0.25	2.97 ±0.050 NS	4.75 ±0.278 NS
glutamate n = 4	1.86 ±0.207	15.95 ±0.47	2.86 ±.031	8.86 ±0.87	2.11 ±0.12 NS	21.39 ±0.24 P<0.001	2.86 ±0.046 NS	10.23 ±0.629 NS
α-ketoglutarate + malonate n = 4	2.27 ±0.24	10.27 ±0.76	3.80 ±0.038	4.74 ±0.753	2.98 ±0.21 NS	15.66 ±1.02 P<0.01	3.84 ±0.069 NS	5.48 ±0.323 NS

Table III: increases in the absolute and percentage rates of respiratory states 3 and 4.

		increase in state 4 respiration		increase in state 3 - respiration	
		Absolute increase (µg atoms O/hr.mg)	percentage increase %	Absolute increase (µg atoms O/hr.mg)	percentage increase %
FAD-linked Substrates	α-glycerophosphate	1.24	34	2.99	68
	succinate	1.63	14	3.85	14
NAD-linked Substrates	α-ketoglutarate	0.75	25	5.72	30
	Pyruvate++Malate	0.70	17	5.47	30
	glutamate	NS	NS	5.44	34
	α-ketoglutarate malonate	NS	NS	5.39	52

respirations are more pronounced for the FAD-linked substrates than for the NAD-linked substrates. A look at table II can explain this. The respiratory control ratios for the FAD-linked substrates are relatively much less than those for the NAD-linked substrates. In other words, the rates of respiration in the absence of exogenous ADP (state 4), are relatively high. This implies that the tightness of coupling at the second and third phosphorylation sites may be relatively weaker than that at the first site. In fact, Christiansen et al., (1969) suggested that in uncoupled brown adipose tissue mitochondria site I is the least uncoupled. Therefore, in the absence of ADP, an increase in the activities of the dehydrogenases of the FAD-linked substrates or an increase in the amounts of some components of the electron transport system could be reflected by a greater increase in respiration than with the NAD-linked substrates since the oxidation of the latter is more limited by the availability of ADP to site I.

It is also noticed in table III that the increases in state 3 respiration for the NAD-linked substrates are more pronounced than those for the FAD-linked substrates. This can be explained by the possibility that ADP which becomes available in state 3 will abolish the restriction imposed on the electron transfer at site I, and therefore the rates of respiration for NAD-linked substrates will increase and exceed the increase observed for the FAD-linked substrates. Table III shows that there are increases in the state 3 respiration

rate of all the NAD-linked substrates and the FAD-linked substrates. The increases in state 3 respiration for all the substrates studied are more pronounced than the increases in state 4 respiration. Two possible suggestions can explain these findings. The first is that the mitochondria of the cold-acclimated rat may take up ADP at a faster rate. The second is that there may be increases in any of the following:

i - the activities of the dehydrogenases

ii - the permeability of the mitochondria to the substrates

iii - the amounts of some components of the electron transport system.

The changes found in the respiratory properties of the mitochondria of skeletal muscles of cold-acclimated rats not only support the belief that the skeletal muscles could be the major site for NST, but also support the previous findings of our laboratory. Recently, our laboratory reported a change in the ultrastructure of the mitochondria of skeletal muscles of cold-acclimated rats (Behrens and Himms-Hagen, in press). The mitochondria become smaller in size and more numerous so that the mitochondrial mass and the activity of cytochrome oxidase do not change per gram of muscle. The ultrastructural changes are presumed to reflect changes in functional activity of the mitochondria.

The next step was to find out why the mitochondria of the cold-acclimated rat have higher respiratory rates in both states 3 and 4, and why the increase is more pronounced in state 3. The changes in respiratory enzyme activities are described in section 3. The changes in ADP translocation are described in section 5.

SECTION 3

DETERMINATION OF THE SPECIFIC ACTIVITIES OF SOME KEY ENZYMES OF THE CITRIC ACID CYCLE AND RELATED ENZYMES IN THE SKELETAL MUSCLE MITOCHONDRIA OF COLD AND WARM-ADAPTED RATS.

i - Purpose of the experiment: to find out whether the increases in the rates of states 3 and 4 respirations described in section 2 could be explained by increases in the activities of the mitochondrial dehydrogenases. At the same time, the purpose is to correlate the changes found, if any, to the mechanism of NST.

ii - Description of the experiment:

The mitochondria were prepared as described in the methods. However, they were finally suspended in 0.25 M sucrose. The mitochondrial enzymes studied were:

a) α-glycerophosphate dehydrogenase (FAD)
b) Succinate dehydrogenase (FAD)
c) malate dehydrogenase (NAD)
d) Isocitrate dehydrogenase (NAD)
e) β-hydroxybutyrate dehydrogenase (NAD)
f) glutamate dehydrogenase (NAD)
g) α-ketoglutarate dehydrogenase (NAD)

The steps followed in the experiment were the following:

1 - The medium in which the activities of the above enzymes were measured consisted of one of the substrates 50 mM pH = 7.4; KCN, 1 mM; KH_2PO_4, 50 mM, pH 7.4; NAD, 0.1 μM in the

case of the NAD-linked substrates; the electron acceptors phenazine methosulphate (PMS) and iodonitrotetrazolium violet (INT), 0.025% and 0.1% respectively; between 10 and 80 µg of mitochondrial protein in a total volume of 1 ml. (Nachlas et al., 1960), (Singer and Lusty, 1960), (Lee and Lardy, 1965), (Gardner, 1974).

2 - The PMS-INT mixture is very unstable and sensitive to light. Therefore it was prepared just before use. The reactions were carried out under dark conditions (using only dim lights from some distant lamps).

3 - After 10 minutes of temperature equilibration at 37°C, the PMS-INT mixture was added. The reaction was stopped at different time intervals (0, 5, 10 and 15 minutes) after the addition of PMS-INT by the addition of 1 ml of 10% TCA. The formazan formed was extracted by vortexing with 4 ml. of ethyl acetate in the cases of α-glycerophosphate, succinate and malate, but with 2 ml. of ethyl acetate in the case of the remaining substrates in order to get a higher reading. Then, a sample was taken from the top layer and its absorbance was read at wavelength 490 against a blank which lacked only the substrate.

When β-hydroxybutyrate dehydrogenase was assayed, the mitochondrial suspension had to be sonicated for 15 seconds on salted ice at medium intensity using the Biosonik III (Bronwill Scientific). The sonication was found to be

essential for the detection of activity.

When isocitrate dehydrogenase was assayed, 2×10^{-3} M ATP was present for activation of the enzyme (Lee and Lardy, (1965).

iii - Results of discussion:

The results are shown in table IV. As can be seen in this table, only two enzymes changed, the specific activity of the mitochondrial (FAD) α -glycerophosphate dehydrogenase increased by 60% and that of the mitochondrial (NAD) malate dehydrogenase decreased by 24%. What is the significance of these findings:

The mitochondrial α-glycerophosphate dehydrogenase seems to play a major role in intermediary metabolism. In skeletal muscles for example, a high activity of this enzyme is found in the white fast-twitch-glycolytic fibers which have a high anaerobic activity; and a low activity in the red slow-twitch-aerobic, low glycolytic fibers. Other fibers have a high aerobic capacity in addition to high glycolytic capacity, and are found to contain high activities of the enzyme (Peter et al, 1972). Therefore it seems that there is a correlation between the activity of this enzyme and the activity of the pathway of glycolysis. Consistent with this conclusion is the finding by Bass et al., (1969) that the ratio of the mitochondrial α -glycerophosphate dehydrogenase to the activity of the pathway of glycolysis is constant in all the muscle types investigated. Therefore, a measure of the relative activity

Table IV: The specific activities of some key enzymes of energy metabolism in the mitochondria of skeletal muscle of cold and warm-acclimated rats.

Enzyme	No. of Exp.	Specific activities Δ O.D$_{490}$/min/mg mitochondrial protein	
		Warm	Cold
β-hydroxybutyrate dehydrogenase	5	0.0470 ±0.0005	0.0475 ±0.0009 NS
glutamate dehydrogenase	5	0.0546 ±0.0011	0.0514 ±0.0019 NS
isocitrate dehydrogenase	5	0.100 ±0.003	0.102 ±0.003 NS
α-glycerophosphate dehydrogenase	5	0.365 ±0.009	0.583 ±0.013 t=13.64 p<0.001
malate dehydrogenase	5	0.861 ±0.03	0.654 ±0.025 t= 5.35 p<0.001
succinate dehydrogenase	5	1.469 ±0.089	1.327 ±0.044 t=1.44 NS
α-ketoglutarate dehydrogenase	5	0.123 ±0.002	0.124 ±0.002 NS

of the enzyme can be used as a marker of the relative rate of glycolysis.

The mechanism by which this enzyme can control glycolysis is through its role in the α-glycerophosphate shuttle in transporting reducing equivalents from the cytoplasm into the mitochondria. A high activity of the enzyme implies a high $NAD/NADH_2$ ratio in the cytoplasm, and the rate of glycolysis (Kornacker and Ball, 1968). Consistent with this mechanism is the correlation between the activity of this enzyme and the activity of lactate dehydrogenase (Peter et al., 1972; Bass et al., 1969). The correlation is believed to be due to the dependence of lactate dehydrogenase on the cytoplasmic concentration of NAD. Isaacs et al., (1969) showed that the heart muscle can oxidize lactate more when the α-glycerophosphate shuttle is more active.

The mitochondrial α-glycerophosphate dehydrogenase can also play an important role in lipid metabolism. Since α-glycerophosphate is a substrate for the synthesis of glycerophosphatides and tryglycerides, an enzyme that can oxidise this substrate must play a key role. This has been shown in the brown adipose tissue which is known to have a high metabolic rate (Ohkawa et al., 1969). The brown adipose tissue was found to contain an unusually high activity of the enzyme, 10 times that in liver, 20 times that in the white adipose tissue and 9 times that in the kidney. The authors

suggest that the apposition of the lipid droplets to mitochondria observed in brown fat cells by Napolitano and Fawcett (1958) may be a morphologic expression of the key role of this enzyme in regulating the availability of α-glycerophosphate for fatty acid esterification.

On the other hand, the activity of the mitochondrial α-glycerophosphate dehydrogenase seems to correlate with the metabolic rate. In the brown adipose tissue for example, Barnard et al., (1970) reported a parallel increase in the enzyme's activity with the respiratory activity of the tissue after birth and after cold-acclimation. Also, Smith (1964) and Smith and Hoijer (1962) reported that the only appreciable enzymatic change observed in the liver and brown adipose tissues after cold-acclimation was an increase in the activity of the mitochondrial α-glycerophosphate dehydrogenase.

The enzyme is also reported to increase in hyperthyroidism. Lee and Lardy (1965) reported a 20-fold increase in the activity of the enzyme in the liver after thyroid hormone administration. Kubista et al., (1971) reported an increase in this enzyme in heart and red skeletal muscles after thyroid hormone administration. The enzyme did not increase in the white muscles possibly beacuse the enzyme's activity is already high in the white muscles (Peter et al., 1972).

Therefore, there seems to be a correlation between the activity of the mitochondrial α-glycerophosphate dehydrogenase,

glycolysis and hyper-metabolism. The finding in this work that there is a 60% increase in the specific activity of the enzyme in the skeletal muscle mitochondria of cold-acclimated rats may indicate that:

a) There is an increased capacity for glycolysis in the skeletal muscles of cold-acclimated rats. In this connection Depocas showed that nonfasting (1960C) and fasting (1962) rats increase their glucose oxidation after exposure to cold and during cold-acclimation in proportion to the increase in respiratory CO_2 production. In addition, Depocas showed that in both fasting and nonfasting rats, the glucose concentration in the plasma, the volume distribution of glucose and the turnover of glucose increase after cold exposure and cold acclimation.

b) The skeletal muscles of the cold-acclimated rat have a greater capacity to consume oxygen than those of the warm-acclimated rat. This finding gives support to the beief that the skeletal muscles contribute significantly to NST.

The mitochondrial α-glycerophosphate dehydrogenase might be activated in other cases of hypermetabolism. This enzyme was found to be activated by Ca^{2+} in the liver mitochondria (Bukowiecki and Lindberg, 1974). Thus, in shivering and exercise where the metabolic rate increases, the Ca^{2+} that leaks into the cytosol from the endoplasmic retriculum as a result of the nervous impulses might activate the enzyme which in turn might allow the speeding up of the

rate of glycolysis in order to deliver substrates at a faster rate to the rapidly phosphorylating mitochondria. In addition, Hansford and Chappell (1967) reported that this enzyme is activated by Ca^{2+} in blowfly flight-muscle mitochondria. The authors suggested from kinetic data that the activation of the enzyme was through the increase in the affinity of the enzyme for substrate.

No changes were found in the specific activities of the mitochondrial dehydrogenases, succinate and β-hydroxybutyrate. Succinate dehydrogenase is known to be localized in the inner membrane (Sottocasa et al., 1967). β-hydroxybutyrate dehydrogenase, on the other hand, is known to be localized on the inside of the inner membrane (Matlib and O'Brien, 1975).

No changes were found in the specific activities of the dehydrogenases α-ketoglutarate and isocitrate (NAD), the two enzymes being known to be loosely bound to the inner membrane (Matlib and O'Brien, 1975).

In the case of the dehydrogenases for glutamate and malate, two "true" matrix enzymes (Matlib and O'Brien, 1975), no change was found in the specific activity of glutamate dehydrogenase, but a 24% decrease in that of malate dehydrogenas

Therefore, no increases in enzyme activities were found (except for α-glycerophosphate dehydrogenase), which could explain the higher rates of states 3 and 4 respiration observed in section 2. The increases in states 3 and 4 respiration could then be attributed to either an increase

in ADP uptake or to an increased permeability to the substrates.

The 24% decrease in the specific activity of the mitochondrial malate dehydrogenase cannot be explained at this stage. This enzyme is known to be the most available in the cell in terms of concentration, is believed to have the fastest turnover of all the mitochondrial enzymes, and was found to decrease by 35% in disuse atrophy of skeletal muscles (Max et al., 1973). The authors suggested that this enzyme is the first to reflect changes in the mitochondria during disuse atrophy. With these facts in mind, it was suspected that the 24% decrease in the specific activity of the enzyme might reflect a decreased matrix protein content per mg mitochondrial protein, and thus an increased membrane protein content per mg mitochondrial protein. This possibility is strengthened by the findings of Barnard et al., (1970). The authors showed that the mitochondria of the brown adipose tissue of cold-acclimated rats have more inner membrane relative to the matrix. They suggested that the high metabolic rate of this tissue in the cold-acclimated rat might be due to an increase in the components of the electron transport chain. Although no changes were found in the specific activities of the inner-membrane-bound succinate and β-hydroxybutyrate dehydrogenases, yet a 60% increase in the specific activity of the inner membrane-bound metachondrial α-glycerophosphate dehydrogenase was found. In addition to this, Beyer et al., (1962) reported an increase

in coenzyme Q levels in the skeletal muscles of cold-acclimated rats; Klain (1963) reported an increase in cytochrome C content per gram skeletal muscle. Both, cytochrome C and coenzyme Q are localized in the inner membrane. Therefore, it was necessary to assess the distribution of mitochondrial proteins in the matrix and membrane fractions of the skeletal muscle mitochondria. This is described in section 4.

On the other hand, the mitochondrial malate dehydrogenase is involved in the malate-aspartate shuttle for the transport of reducing equivalents across the mitochondrial membrane. The 24% decrease found in the specific activity of this enzyme indicated that there might be an alteration in the operation of this shuttle. This is described in section 5.

SECTION 4

STUDY OF THE RELATIVE DISTRIBUTION OF MITOCHONDRIAL PROTEIN IN THE MATRIX AND MEMBRANE FRACTIONS OF THE SKELETAL MUSCLE MITOCHONDRIA OF COLD AND WARM-ACCLIMATED RATS.

1. Purpose of the experiment:

In order to study some enzymes of the malate-asparate shuttle (section 5), it was necessary to fractionate the mitochondria into matrix and membrane fractions, and to assess the distribution of mitochondrial protein in these fractions and whether this distribution was altered by cold-acclimation.

On the other hand, the finding reported in section 3 of an increase in the specific activity of the inner membrane-bound α-glycerophosphate dehydrogenase, and a decrease in a "true" matrix enzyme namely, malate dehydrogenase implied that there might be relative changes in the protein contents of both matrix and membrane fractions per unit mitochondrial protein. Other components of the inner membrane were found to be increased by cold-acclimation, namely, coenzyme Q (Beyer et al., 1962), and cytochrome C (Klain, 1963) while some of the inner-membrane components were found not to change such as succinate dehydrogenase and β-hydroxybutyrate dehydrogenase (this work). In addition, no change was found in the specific activity of cytochrome oxidase (Himms-Hagen et al., 1975).

ii - Description of the experiment:

The mitochondria were isolated and fractionated into the membrane and matrix fractions as described in the methods.

iii - Results and discussion:

The results are shown in table V. No changes were found in any of the following:

a) the recovery of whole mitochondrial protein.

b) the recovery of matrix proteins.

c) the recovery of membrane proteins.

It must be noted however, that the sonication method employed here could have dislocated the relatively loosely-bound protein components of the inner membrane so that these components were recovered in the matrix fraction, thus masking the situation existing in vivo. However, the conclusion that can be stated here is that there is no change in the bulk tightly-bound protein content of the membrane fraction after cold-acclimation.

Table V. The relative membrane and matrix protein contents in mitochondria.

Fraction	Cold	Warm
Wet muscle	5 grams	5 grams
Whole mitochondria (mg)	6.325 ± 0.549 NS	6.704 ± 0.592
Matrix (mg)	1.653 ± 0.074 NS	1.821 ± 0.242
Membrane (mg)	3.331 ± 0.258 NS	3.625 ± 0.439
% Matrix /Mitochondria	26.37% ±1.57% NS	26.69% ±0.967%
% Membrane /Mitochondria	52.74% ±0.63% NS	52.80% ±0.818%
% Matrix /Membrane	49.73% ±2.148% NS	49.94% ±1.035%
Recovery: % Matrix + Membrane /Mitochondria	79.11% ±2.2% NS	79.49% ±1.527%

n = 4

SECTION 5

DETERMINATION OF THE SPECIFIC ACTIVITIES OF THE ENZYMES INVOLVED IN THE SHUTTLES RESPONSIBLE FOR THE TRANSFER OF REDUCING EQUIVALENTS ACROSS THE MITOCHONDRIAL MEMBRANES.

i - Purpose of the experiment:

The only enzyme changes found in section 3 were two enzymes involved in two shuttles responsible for the transport of reducing equivalents from the cytosol into the mitochondrion. This finding prompted a more detailed study of the shuttles.

Since the inner membrane of the mitochondrion is relatively impereable to pyridine nucleotides (Purvis and Lowenstein, 1961), the transfer of reducing equivalents from the cytosol to the mitochondrion is believed to occur indirectly by way of shuttles. Two such shuttles are the α-glycerophosphate and the malate shuttles (Bucher and Klingenberg, 1958).

In the α-glycerophosphate shuttle, dihydroxyacetone phosphate oxidizes NADH in the cytosol a reaction catalyzed by an NAD-linked cytoplasmic α-glycerophosphate dehydrogenase. α-glycerophosphate then enters the mitochondrion and is reoxidized to dihydroxyacetone phosphate, a reaction catalyzed by the flavin-linked α-glycerophosphate dehydrogenase which is localized on the outside of the inner membrane. In discussing the thermogenic role of the α-glycerophosphate shuttle proposed by Smith (1964), it was suggested by the author that the

mitochondrial α-glycerophosphate dehydrogenase is linked to cytochrome C. In fact, it is not known yet whether the mitochondrial α-glycerophosphate dehydrogenase is linked to cytochrome C or FAD.

In the original malate shuttle, oxaloacetate is reduced in the cytoplasm to malate catalyzed by the cytoplasmic malate dehydrogenase. Malate then enters the mitochondrion where it is reoxidized to oxaloacetate by the mitochondrial malate dehydrogenase. Oxaloacetate then diffuses back to the cytosol. However, a negative side of this simple malate shuttle is the fact that the inner mitochondrial membrane is poorly permeable to oxaloacetate (Haslam and Krebs, 1968). Therefore Borst (1963) modified the malate shuttle to include transamination between oxaloacetate and glutamate by means of the cytosolic and mitochondrial aspartate aminotransferase isoenzymes.

Therefore the study of the activities of all those enzymes involved in the two shuttles was necessary. The α-glycerophosphate shuttle is composed of two enzymes, the cytoplasmic and the mitochondrial α-glycerophosphate dehydronenase. The mitochondrial enzyme was found to increase in specific activity by 60%. In this section the activity of the cytoplasmic enzyme will be described.

In the case of the malate-aspartate shuttle, only the mitochondrial malate dehydrogenase has been studied and a 24% decrease was found. Therefore, in this section, the activities

of all the enzymes involved in the malate-aspartate shuttle will be studied including a repeated study of the mitochondrial malate dehydrogenase, but employing a different method just for comparison.

ii - Description of the experiments

a) The cytoplasmic α -glycerophosphate and malate dehydrogenases.

The cytoplasmic fraction was prepared as described in the methods. The enzyme activities were measured spectrophotometrically by following the increase in absorbance at 340 nm due to the reduction of NAD. The reaction medium contained the following: glycine (NAOH) 0.1 M pH 10.0. NAD 0.5 mM, substrate (α -glycerophosphate or malate) 5 mM, enzyme preparation (the cytoplasmic fraction), 15, 20 and 25 μ g protein. This protein content was used so that the increase in absorbance at 340 nm was less than 0.06/minute. The total volume of the reaction medium was 1 ml. The blank used contained all the components except NAD. The temperature of the reaction was room temperature (22-25°C), (Lee and Lardy, 1965).

The reaction was started by the addition of the substrate after NAD reduction by endogenous substrates had stopped. This is time zero, and then readings were taken every 15 seconds. The rate was linear for at least one minute.

The extinction coefficient for NADH is 6.22 $mM^{-1}\ cm^{-1}$.

b) The cytoplasmic alanine and aspartate aminotransferases

The cytoplasmic fraction was prepared as described in the methods. The enzyme activity was studied by following the decrease in $O.D_{340}$ due to the oxidation of NADH at room temperature. The reaction medium contained the following: KH_2PO_4 0.1 M pH 7.3, substrate (alanine 20 mM or aspartate 10 mM), NADH 0.2 mM, α-ketoglutarate 4.1 mM, lactate dehydrogenase 2 units (when alanine is the substrate), or malate dehydrogenase 2 units (when aspartate is the substrate), enzyme preparation (15, 20, 25 µg protein when aspartate is the substrate; and 100, 150, 200 µg when alanine is the substrate).

The reaction was started by the addition of α-ketoglutarate. The rates were linear for at least 3 minutes, and readings were taken every one minute (Karmen, 1955; Wroblewski and La Due, 1955).

The extinction coefficient for NADH is 6.22 mM^{-1} cm^{-1}.

c) The mitochondrial aspartate aminotransferase and mitochondrial malate dehydrogenase.

The mitochondria were isolated as described in the methods. The preparation of the soluble matrix and the membranes of the mitochondria is also described in the methods.

The assay method used for the mitochondria aspartate aminotransferase was the same as that used for the cytoplasmic one. The assay method used for the mitochondrial malate dehydrogenase was the same as that used for the cytoplasmic one.

The enzyme preparations used for the assay of the above two enzymes were two for each enzyme: One was from the matrix preparation and the other from the membrane preparation. 5 µg of matrix preparation was used in the case of both enzymes, while 50 µg membrane protein was used for ech enzyme.

iii) Results and Discussion:

As shown in table VI, no changes were found in the specific activities of the cytoplasmic malate and α-glycerophosphate dehydrogenases, nor in the specific activities of the cytoplasmic alanine and aspartate aminotransferases. Also, no change was found in the specific activity of the mitochondrial aspartate aminotransferase, a matrix enzyme (Matlib and O'Brien, 1975).

On the other hand, a 19% decrease in the specific activity of the mitochondrial malate dehydrogenase was found in the matrix. This 19% decrease supports the finding in section 3 of a 24% decrease in the specific activity of this enzyme when employing whole mitochondria as the enzyme preparation and a different assay method.

Thus, only two enzymic changes were found in the enzyme components of the two shuttles. These two enzymes are the mitochondrial α-glycerophosphate dehydrogenase and the mitochondrial malate (NAD) dehydrogenase. The former enzyme increased by 60% (section 3), and the latter decreased by 24% (section 3) and 19% (section 5). Thus, the membrane and matrix

Table VIa: The cytoplasmic α-glycerophosphate and malate dehydrogenases.

nmoles NADH formed/min/mg

	α-glycerophosphate dehy.	malate dehy.
Cold	216 ±12.02	395 ±14.45
Warm	205 ±5.09 NS	408 ±26.85 NS

n = 3

Table VIb: The cytoplasmic alanine and aspartate aminotransferases.

nmoles NADH formed/min/mg

	Alanine aminotrans.	Aspartate Aminotrans.
Cold	20.89 ±3.26	441 ±18.24
Warm	20.49 ±2.3 NS	460 ±20.58 NS

n = 4

Table VIc: The mitochondrial aspartate aminotransferase and malate dehydrogenases.

μ moles NADH/min/mg

	Aspartate aminotransferase		Malate dedydrogenase	
	Matrix	Membrane	Matrix	Membrane
Cold	3.12 ±0.1889	0.126 ±0.037	5.35 ±0.261	o.390 ±0.106
Warm	3.2 ±0.2 NS	0.126 ±0.032 NS	6.60 ±.187 .01<p<.02	0.454 ±0.079 NS

n = 4

fractions of the mitochondria do not change as a unit; rather the changes can be very specific in each fraction. Examples to this statement are:

a) The "true" matrix enzymes glutamate dehydrogenase and aspartate aminotransferase did not change.

b) The inner membrane-bound dehydrogenases succinate and β-hydroxybutyrate did not change.

Since it is generally accepted that the amount of the mitochondrial α-glycerophosphate dehydrogenase determines the rate of operation of the α-glycerophosphate shuttle, it was concluded (see section 3 in discussion) that the α-glycerophosphate shuttle is capable of operating more rapidly which in turn might lead to an increased rate of glycolysis. In the case of the malate-aspartate shuttle, it is not known whether the amount of mitochondrial malate dehydrogenase is the main limiting factor in the operation of the shuttle, but this possibility seems unlikely for the following reasons:

a) The operation of the malate-aspartate shuttle requires a rapid and easy passage of its substrates, malate, glutamate, α-ketoglutarate and aspartate. Thus, any factor that can potentially inhibit this process, can be rate-limiting.

b) The operation of the malate-aspartate shuttle requires that sufficient amounts of its substrates are present all the time in the cytoplasm and the mitochondria. The competition between the different metabolic pathways in the cell on these substrates would influence the rate of operation of the shuttle.

c) The operation of the malate-aspartate shuttle requires that all the enzymic components of the shuttle (cytoplasmic and mitochondrial) be relatively active, all at the same time. The ever-changing environment of the cell can influence any of these enzymes and in turn influence the rate of operation of the shuttle.

Thus, the significance of the decrease in the specific activity of the mitochondrial malate dehydrogenase in the skeletal muscles of cold-acclimated rats remains to be explained.

In conclusion no enzymic changes were found that could explain the increases in states 3 and 4 rates of respiration except for the increase in mitochondrial α-glycerophosphate dehydrogenase. In the next section (section 6) the possibility that an increase in the rate of ADP uptake may be the cause, will be investigated.

SECTION 6

DETERMINATION OF THE ACTIVITY OF THE ADENINE NUCLEOTIDE TRANSLOCATION ACROSS THE MITOCHONDRIAL MEMBRANES OF SKELETAL MUSCLES OF COLD AND WARM-ADAPTED RATS.

i - Purpose of the experiment:

Since no enzymic changes were found that could explain the increases in the rates of states 3 and 4 respirations reported in section 2 (except for α-glycerophosphate), then the possibility of an increased rate of ADP uptake by the skeletal muscle mitochondria of cold-adapted rats had to be investigated.

ii - Descritpion of the experiment:

The mitochondria were isolated as described in the methods, but the last homogenization was done in 0.25 M sucrose to end with a protein concentration of 0.5 mg protein per 50µl. Then:

140µl of a medium containing the following:

K_2HPO_4	13 mM	pH = 7.4
KH_2PO_4	3 mM	
KCl	58 mM	
NaCl	30 mM	

was added to a small test tube. Then 50µl of the mitochondrial suspension (0.5 mg) in 0.25 M sucrose was added. The reaction was started by adding 10µl of an ADP solution containing the following:

0.04µmoles ADP
0.04 µCi (U $-^{14}C$ ADP)

The reaction was carried out at 6°C (Babior et al., 1973). The reaction was stopped after 5 seconds by adding 100 µl of 0.2 mM potassium atractyloside solution. Then, quickly 100µl was withdrawn and injected into the center of a millipore filter (0.45 µm) under reduced pressure. The mitochondria were then quickly washed with 10 ml of 0.15 M Na Cl solution. The whole operation starting from the addition of ADP to the Na Cl washing took less than 30 seconds.

The millipore filter was then removed, dissolved in a Beckman Bio-Solv BBS-3, special dissolution agent and toluene containing PPO and dimethyl POPOP and the amount of radioactivity was counted in a Beckman LS-250 liquid scintillation counter, using the external standard ratio method, in order to calculate the amount of ADP taken up by 0.5 mg of mitochondrial protein. The counts per minute were converted to disintegrations per minute and from this the total amount of ADP taken up by the mitochondria was calculated by multiplying the percentage of radioactivity taken up by the mitochodnria, by the total amount of ADP present initially in the test tube (radioactive + nonradioactive).

The nonspecific binding of ADP was estimated in the same way except that atractyloside was present before starting the reaction with ADP.

For a given set of mitochondria, the total ADP uptake was determined in duplicate, and nonspecific uptake was determined

once. The carrier-mediated ADP transport was calculated by subtraction of the value of non-specific ADP uptake from the average of the values for total ADP uptake. The non-specific ADP uptake represented generally less than 30% of the total ADP uptake.

iii - Results and discussion:

The results are shown in table VII. As can be seen in the table, there is a 35% increase in the rate of ADP uptake by the skeletal muscle mitochondria of cold acclimated rats.

From the kinetic data, Klingenberg and Pfaff (1966) showed that the adenine nucleotides enter two compartments of rat liver mitochondria, the first being the intermembrane space which the adenine nucleotides enter passively; the second being the matrix compartment within the inner membrane which they suggested contains the atractyloside-sensitive carrier. In their studies, Winkler et al., (1968) showed that mitochondrial preparations stripped from their outer membrane had an atractyloside-sensitive ADP uptake which was very similar to that of intact mitochondria in rate and capacity. Their conclusion was that the inner membrane contains the adenine nucleotide translocase. It is not yet known exactly where this enzyme complex is localized in the inner membrane, whether on the outer or inner surface.

The finding here of a 35% increase in the rate of ADP uptake by the skeletal muscle mitochondria of cold-acclimated rats is a very important one since it appears that there is a correlation between the rate of mitochondrial ADP uptake

Table VII

Carrier-mediated ADP-uptake

	nmoles ADP uptake /min/mg	
Cold n = 4	1.78 ± .037	$P < 0.01$
Warm n = 4	1.32 ± .08	

and the metabolic rate. First of all, it is believed that the number of atractyloside-sensitive nucleotide binding sites in the mitochondria must be relatively small compared to the total number of nucleotide molecules in the mitochondria, which means that the amount of enzyme complex must be a limiting factor in the entrance of ADP into the mitochondria. Bruni et al., (1965) calculated the number of atractyloside-binding sites in rat liver mitochondria and found that about 0.15 n moles of atractyloside were bound per mg of mitochondrial protein, and that 2% of the total mitochondrial adenine nucleotide would be bound to atractyloside-sensitive sites at a given time. Therefore, the 35% increase in the rate of ADP uptake found here could lead to increased oxygen consumption in coupled mitochondria, since ADP is the prime regulator of oxygen consumption in coupled mitochondria.

On the other hand, it was shown that the rate of uptake of ADP by the mitochondria is directly proportional to the metabolic rate. For example, Shrago et al., (1972) showed that the liver mitochondria of the hibernating ground squirrel (low metabolic rate), respond very weakly to the addition of ADP due to the low rate of ADP uptake by these mitochondria. The authors suggested that the acyl CoA esters of free fatty acids which accumulate in liver during hibernation, inhibit the adenine nucleotide translocase as evidenced by the finding that carnitine can release the inhibition

and that the liver mitochondria of the diabetic rat and monkey (diabetes is a condition where fatty acid oxidation is stimulated) are also uncoupled and show a low rate of ADP uptake. On the other hand. Babior et al., (1973) showed that in hyperthyroidism, there is an increased activity of the adenine nucleotide translocase in the liver mitochondria of the rat (hyperthyroidism is a condition where the metabolic rate is high). Therefore, it becomes very probable that the activity of the adenine nucleotide translocase is a prime determinant of the metabolic rate. The finding of a 35% increase in the rate of ADP uptake in the skeletal muscle mitochondria of cold-acclimated rats (a condition where the metabolic rate is high) supports this belief.

The 35% increase in the rate of ADP uptake found here could well explain the increased rates of state 3 respiration for all the substrates studied in section 2, except for α-glycerophosphate in which case the 60% increase in the specific activity of the mitochondrial α-glycerophosphate dehydrogenase was the prime cause. However, the increased rate of ADP uptake could explain the observation that the increase in state 3 respiration with α-glycerophosphate (68%) was slightly greater than the increase in α-glycerophosphate dehydrogenase (60%).

A very important question now arises: If the rate of ADP uptake determines the metabolic rate, then what about

the rate of ADP uptake during shivering and exercise, conditions where the oxygen consumption of the animal is as high as in cold-acclimation ?

It is possible that the rate of ADP uptake also increases during shivering and exercise. However, the increase in these two conditions might be due to activation of this enzyme. During shivering and exercise, the Ca^{2+} concentration in the cytoplasm rises as a result of the nervous impulses and depolarization of the cell membranes. Bygrave and Spencer (1972) showed that Ca^{2+} can stimulate the translocation of ADP. From kinetic data, the authors showed that Ca^{2+} increases both the affinity for adenine nucleotides and the maximal velocity of the translocase. The authors suggest that this increased activity results from an interaction of Ca^{2+} with phospholipids located near the translocase enzyme. In shivering and exercise the rate of ADP uptake would increase because the translocase is activated by Ca^{2+} and is working at maximum capacity.

The suggestion that during shivering or exercise of warm-acclimated rats, the rate of ADP uptake by the mitochondria increases due to the activation of the ADP translocase by Ca^{2+}, and the finding in this work that the specific activity of the ADP translocase increased by 35% after cold-acclimation, could explain the observations of Hart and Jansky (1963). These authors showed that in the warm-acclimated rat, shivering and exercise do not summate in terms of the increase in heat

production, while in the cold-acclimated rat, exercise and NST do summate. A possible explanation of these observations is the following:

a) In the warm-acclimated rat, both shivering and exercise activate the ADP translocase through the release of Ca^{2+} into the cytoplasm. Thus, since both conditions influence the ADP translocase by the same way, they do not summate because there is a limit after which the ADP translocase cannot be further activated even by further increasing the amount of Ca^{2+} in the cytoplasm.

b) In the cold-acclimated rat, exercise and NST can summate because each influences the enzyme in an independent way. Exercise activates the enzyme through the release of Ca^{2+}, and NST is associated with the presence of more enzyme.

CHAPTER D

GENERAL CONCLUSION AND DISCUSSION

The results of this research can be discussed in terms of three major aspects:

Aspect A: The extent to which the results contribute to the understanding of the mechanism of NST in skeletal muscle.

Aspect B: The possibility that the changes found in this research in the skeletal muscle mitochondria of cold-acclimated rats are produced by the thyroid hormones.

Aspect C: The possibility that the thyroid hormone is one of the components of the mechanism of NST.

Aspect A

The finding that the mitochondria isolated from the skeletal muscles of cold-acclimated rats have normal ADP/O ratios and normal respiratory control ratios rules out uncoupling as the mechanism of NST in skeletal muscles, and places the mechanism of NST in the category of increased energy - consuming pathways and/or processes. This category requires the presence of a complete circuit which can be divided into two half-circuits (see figure 5). The synthesis of ATP is represented by half-circuit 1, and the consumption of ATP is represented by half-circuit 2_n. As shown in the figure half-circuit 2_n can be divided into many sub-half-circuits since energy consuming processes can occur anywhere in the cell, in the cell membrane, in the cytosol and in the organelles.

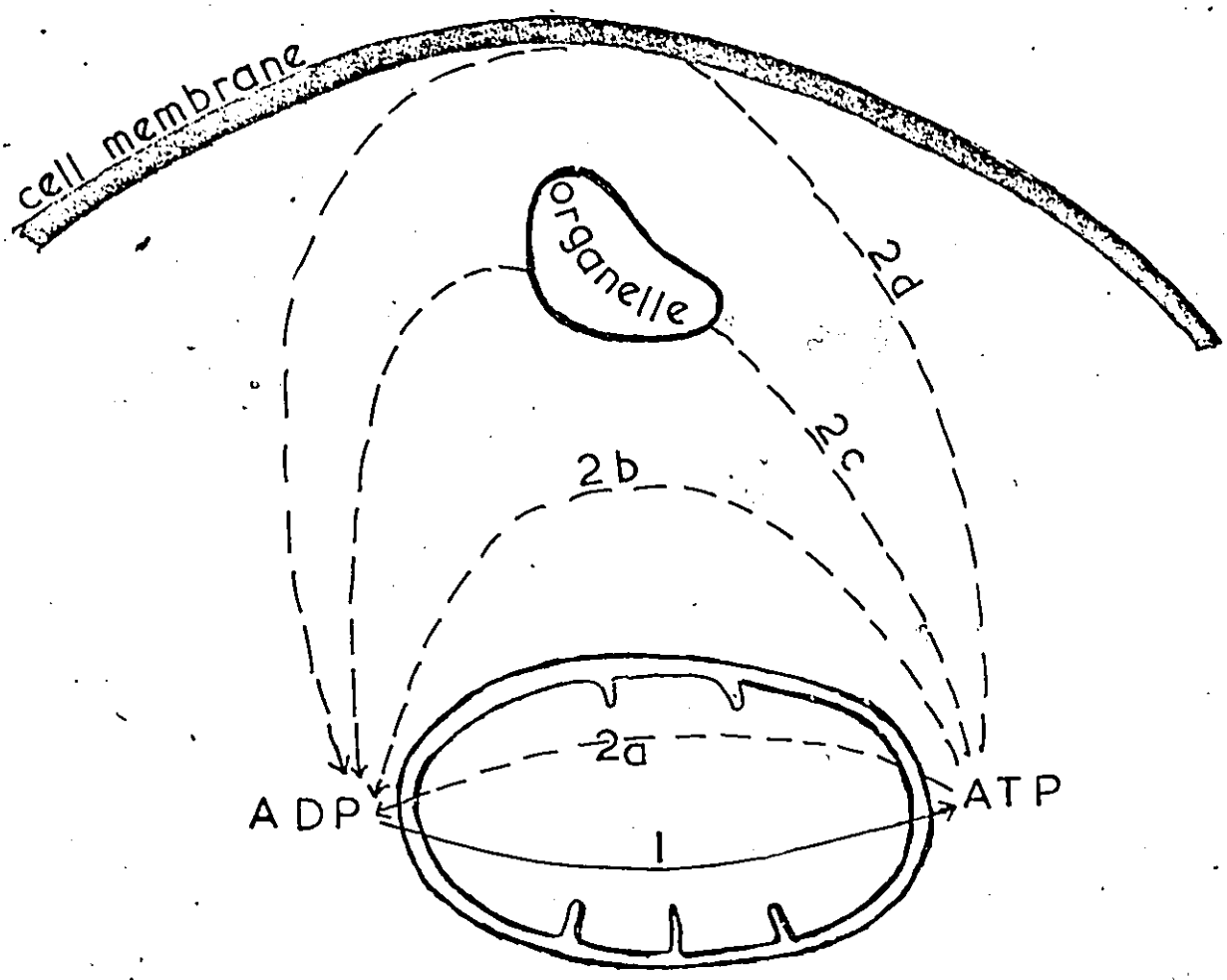


Figure 5. Half-circuits 1 and 2_n.

including the mitochondria. Half-circuit 1 on the other hand, can occur only in the mitochondria.

The significant increases in the activities of the mitochondrial adenine nucleotide translocase and α-glycerophosphate dehydrogenase reported in this thesis suggest that half-circuit 1 might operate at a faster rate in the skeletal muscle mitochondria of cold-acclimated rats.

Both the mitochondrial α-glycerophosphate dehydrogenase (Safer et al., 1971) and the adenine nucleotide translocase (Bruni et al., 1965) are limiting enzymes. The increase in the specific activity of the mitochondrial α-glycerophosphate dehydrogenase might be an indication of an increased capacity of the pathway for glycolysis. In fact, Bass et al., (1969) and Peter et al., (1972) showed that the ratio of the mitochondrial α-glycerophosphate dehydrogenase to the activity of the pathway for glycolysis is constant in all the muscle types investigated. Thus, it seems that the capacity of substrate delivery to the mitochondria of the skeletal muscles of cold-acclimated rats might be augmented.

On the other hand, the increase in the rate of ADP uptake might be an indication of a faster rate of electron transport in the skeletal muscle mitochondria of cold-acclimated rats. In fact, ADP is known to be the prime regulator of the rate of electron transport in coupled mitochondria (Wilson et al., 1974). The possible significance

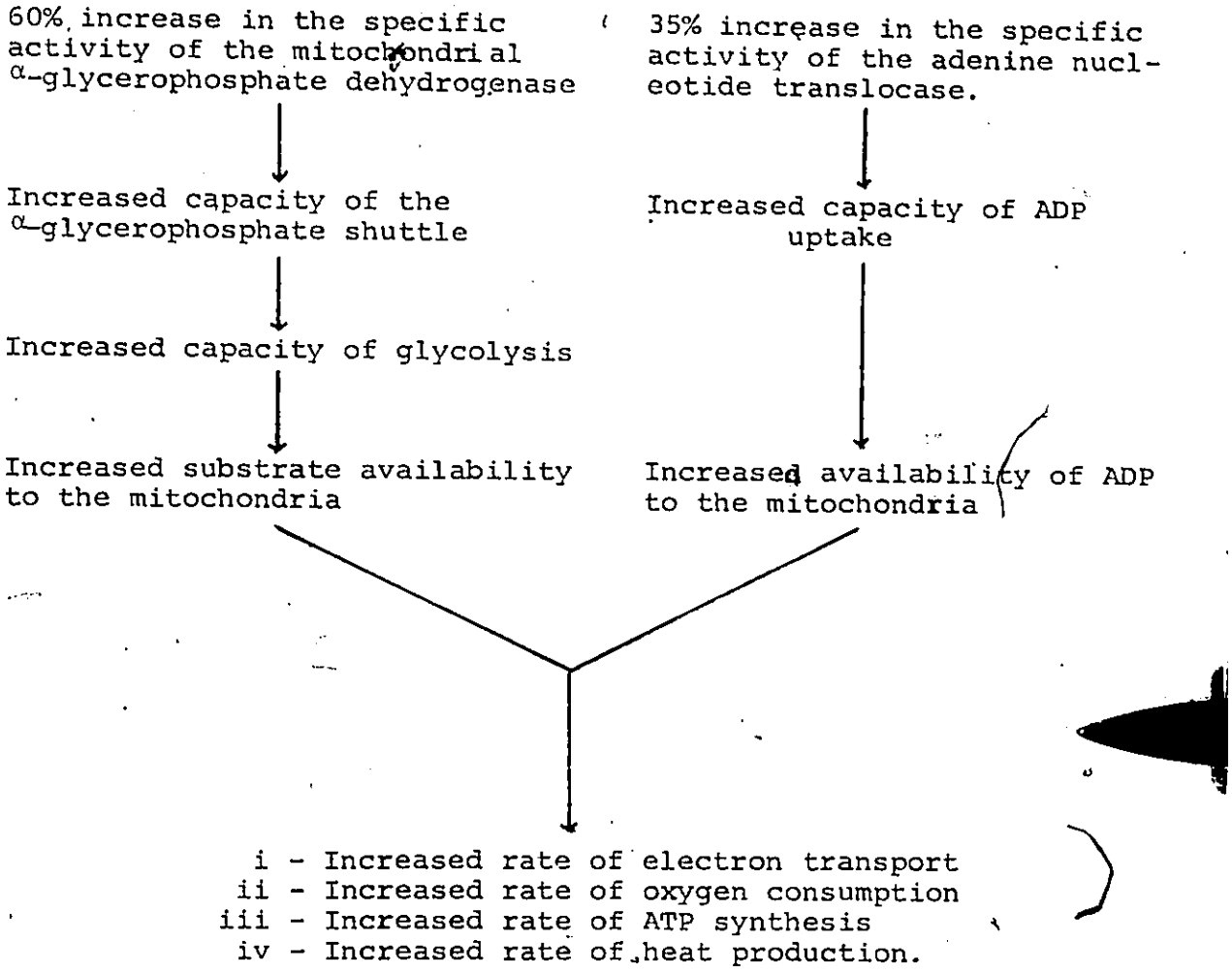
of the increases found in this work in the activities of the mitochondrial α-glycerophosphate dehydrogenase and the adenine nucleotide translocase is summarized in figure 6.

The mitochondrial α-glycerophosphate dehydrogenase (Bukowiecki and Lindberg 1974) and the adenine nucleotide translocase (Bygrave and Spencer, 1972) were shown to be activated by Ca^{2+}. Thus, it becomes possible that these two enzymes might be activated during shivering and exercise where the Ca^{2+} concentration in the cytoplasm rises as a result of the nervous impulses that depolarize the sarcolemma releasing Ca^{2+} from the sarcoplasmic reticulum. If it is assumed that these two enzymes require activation during shivering and exercise (cases where the metabolic rate increases) because the two enzymes are limiting then the increases found in this work in their specific activities might be an indication of their important role in the mechanism of NST through increasing the capacity of half-circuit 1 without a need for activation by Ca^{2+}.

What about half-circuit 2_n ? Since the mechanism of NST was placed under the category of increased energy-consuming pathways and/or processes, then an increase in the utilization of half-circuit 2_n deserves investigation, especially since an increase in the capacity of half-circuit 1 was found. At present this is being investigated in our laboratory.

It must be mentioned however that the increase in the

Figure 6: Significance of the increases in the specific activities of the mitochondrial α-glycerophosphate dehydogenase and the adenine nucleotide translocase.



capacity of half-circuit 1 found in the present work is small relative to the three to four-fold increase in the oxygen consumption observed in the cold-acclimated rat. It is possible that the newly synthesized or unmasked α-glycerophosphate dehydrogenase and adenine nucleotide translocase are more active in vivo, but very sensitive to the isolation method used for the mitochondria so that relatively small increases were detected.

ASPECT B

The changes found in this study on the skeletal muscle mitochondria of cold-acclimated rats are known to be produced in different tissues of the rat after thyroid hormone administration. These changes are the following:

a) After thyroid hormone administration, the mitochondria of rat liver (Smith et al., 1960, 1962; Tata et al., 1963) and skeletal muscle (Tata et al, 1963; Gustafsson et al., 1965; De Groot et al., 1968) show higher respiratory and phosphorylative activities, normal ADP/O ratios and normal respiratory control. Recently Sacktor et al., (1972) and Babior et al., (1973) noted that the rate of state 3 respiration of rat liver mitochondria is more affected by T_3 administration than the rate of state 4 respiration. In the present study the skeletal muscle mitochondria of cold-acclimated rats were found to have normal ADP/O ratios, normal respiratory control ratios, higher respiratory rates and most important, a greater increase in state 3 respiration than in state 4 respiration.

b) After T_3 administration, the specific activity of the adenine nucleotide translocase of the liver mitochondria of rats increases (Babior et al., 1973). A similar increase was found in the skeletal muscle mitochondria of cold-acclimated rats in the course of the present experiments.

c) After thyroid hormone administration, the specific activity of the mitochondrial α-glycerophosphate dehydrogenase increases

in liver (Lee and Lardy, 1965; Israel et al., 1973), in heart muscle (Safer et al., 1971; Kubista et al., 1971), in red skeletal muscles (Kubista et al., 1971), and in the brown adipose tissue of suckling (Hemon and Berbey, 1968) but not older rats (Hemon and Berbey, 1968; Heick et al., 1973). In the present study, a similar increase was found in the specific activity of the enzyme in the skeletal muscle mitochondria of cold-acclimated rats. Cold acclimation also increases the activity of this enzyme in liver (Smith, 1964) and in the brown adipose tissue (Smith, 1964; Barnard et al., 1970; Videla et al., 1975).

The above similar changes might be an indication of an increased utilization of the thyroid hormones in cold-acclimation. However, the cold-acclimated rat is not hyperthyroid in the sense that plasma-bound iodine concentrations are not increased in cold-exposed rats (Rand et al., 1952; and Reichlin et al., 1973) and the basal metabolic rate is not increased (Hsieh, 1963). In addition, Hsieh (1962) showed that the dose of T_4 required for normal growth of rats fed on an iodine-deficient diet containing 0.05% propylthiouracil (PTU) is the same in the cold as in the warm. Thus, it appears that increased amounts of thyroid hormone are not required for cold-acclimation. However, recent research on the utilization of the thyroid hormone in cold-acclimated rats has brought out a different view of the problem. In 1965, Albright et al.,

showed that the T_3/T_4 ratio in all the extrathyroidal of cold-exposed rats has increased. More recently, Balsam (1974) and Balsam and Leppo (1974) showed that the binding of T_3 and T_4 to liver, kidney, spleen, testis and muscle increased in cold-acclimated rats using radioimmunoassays. Reichlin et al., (1973) showed that the plasma T_4 levels were less in cold-adapted than control rats, while plasma T_3 levels were 80% higher. Two explanations could be given to these increases in the T_3/T_4 ratio of the extrathyroidal tissues of cold-adapted rats. The first is that the thyroid gland secretes T_3 in preference to T_4. The second is that there is an increased conversion of T_4 to T_3 by the extrathyroidal tissues of cold-adapted rats. The experiments of Bernal and Rey (1975 a and b) suggest that the latter explanation was correct. These authors showed that in cold-exposed rats, the concentration of T_4 decreased in all tissues, whereas the concentration of T_3 did not decrease. Therefore, the T_3/T_4 ratio of the extrathyroidal tissues of cold-exposed rats increased more than two-fold. They also showed that the activity of the mitochondrial α-glycerophosphate dehydrogenase in a given tissue correlated with the concentration of T_3 and not wiht that of T_4. Thus these authors concluded that the conversion of T_4 to T_3 might play an important role in the expression of this biological effect of T_4, and that this conversion might be increased by cold-exposure.

In this connection, Hillier (1968) showed that there was a positive correlation between the deiodination of thyroxine and the urinary catecholamine excretion in cold-exposed but not in warm-exposed rats. Thus it appears that in the cold-acclimated rat, the increased urinary catecholamine excretion indicates an increased deiodination of T_4 to T_3 by the extrathyroidal tissues. In addition, Sellers et al., (1974) showed that hypothyroid rats can cold-acclimate through a compensatory increase in the activity of the sympathetic nervous system. It is possible that this will lead to a compensatory increase in deiodination.

Thus, if it is assumed that the conversion of T_4 to T_3 plays an important role in the expression of the biological effects of the thyroid hormone and that this conversion is increased in the extrathyroidal tissues by cold-exposure (Bernal and Rey, 1975 a and b) then the specific biochemical increases found in this research could be an indication of an increased utilization of the thyroid hormone. The difference between cold-acclimation and hyperthyroidism is that in the latter, there is increased concentration of circulating hormone due to the injection of large amounts of the hormone by the gland, while in cold-acclimation, there is no increased concentration of circulating hormone, but an increased deiodination of T_4 by the extrathyroidal tissues causing increased T_3/T_4 ratio. Thus in hyperthyroidism and cold-acclimation, there is

increased availability of T_3 to the tissues although by different means, yet leading to similar enzyme changes which might support but could not cause a raised metabolic rate in both conditions.

On the other hand, there are many other biochemical changes known to occur after thyroid hormone administration. Some of these changes were also shown to occur in cold-acclimated rats. For instance:

i - Ismail - Beigi and Edelman (1970, 1971, 1974) showed that the activity of Na-K-ATPase increased in tissue homogenates of liver and kidney after T_3 administration. Recently, Asano et al., (1976) showed that this increase also occurs in skeletal muscles. Videla et al., (1975) showed that the activity of this enzyme doubled in liver of cold-acclimated rats and suggested that the thyroid hormones might be involved in cold-acclimation.

ii - Drabkin (1950) showed that the concentration of cytochrome C increased in liver, heart, kidney and mostly in skeletal muscle of hyperthyroid rats, while it decreased in hypothyroid or thyroidectomized rats. The author suggested that the thyroid hormone might control the tissues' concentration of this electron carrier. Klain (1963) had already shown that cold-exposure increases the concentration of cytochrome C in heart, kidney, lung, spleen, and mostly in liver and skeletal muscles and on this basis, he pointed to the possible

role of the thyroid hormones in cold-acclimation.

iii - Beyer et al., (1961) showed that coenzyme Q levels were increased by thyroid hormone injection and decreased by thyroidectomy in rat liver, skeletal muscle, heart, and kidney, but not in the brain. Later, Beyer et al., (1962) showed that coenzyme levels also increased after cold-acclimation in the same tissues. These authors suggested that the thyroid hormones might be involved in cold-acclimation.

iv - Patton and Platner (1970) compared the effects of cold-acclimation and thyroxine treatment on liver mitochondrial fatty acids. The authors found that both conditions resulted in a decrease in the total unsaturation of fatty acids. On the other hand, when the cold-acclimated rats were injected with an antithyroid drug, this change was prevented. These authors suggested that thyroxine could be responsible for altering the fatty acid composition in the cold-acclimated rats.

It is rather interesting to note that the similar biochemical changes induced by the thyroid hormones and by cold-exposure appear not only in skeletal muscle but in other tissues as well. Thus, it becomes possible that NST might be a generalized phenomenon like the effect of the thyroid hormone on almost all body tissues. In other words, it is possible that all the target cells of the thyroid hormone might be modified during cold-acclimation.

ASPECT C

If it is assumed that the adaptation found in half-circuit 1 is a component of the mechanism of NST (aspect A), and that this adaptation might be a result of an increased utilization of the thyroid hormone (aspect B), then the possibility arises that the thyroid hormone might be one of the components of cold-acclimation. In discussing this possibility, it must be borne in mind that there is much more to cold-acclimation than an increased utilization of the thyroid hormone. The sympathetic nervous system is well established to be a major component of cold-acclimation (see page 11 of the introduction). In addition, more components might be involved such as the nervous system, the cardio-vascular system, the corticosteroids, the growth hormone and so on. Thus, it follows that many facts about the mechanism of NST cannot be explained by an increased utilization of the thyroid hormone. For example, hyperthyroid rats have a permanent elevated basal metabolic rate whereas, cold-acclimated rats can switch on and off a high basal metabolic rate depending on their needs. This regulation of the basal metabolic rate is essential to the understanding of the mechanism of NST. In addition, Hsieh et al., (1966) showed that hyperthyroid rats have increased sensitivity to the calorigenic effect of noradrenaline but no increase in capacity to respond, whereas cold-acclimated rats have an increased capacity to respond.

Moreover, the skeletal muscle mitochondria of cold-acclimated rats become smaller in size but more numerous (Himms-Hagen et al., 1975), whereas in hyperthyroidism, the exact morphological change remains to be settled (see page 52 of the introduction). Thus, caution must be called in interpreting these electron microscopy studies.

To conclude, the results of this work point to a possible involvement of the thyroid hormones in cold-acclimation. However, a study that would prove that the thyroid hormones are a component of NST is one in which the time-course for development of cold-acclimation and deacclimation are compared quantitatively with the time-course for variance of both the T_3/T_4 ratio in the tissues and the biochemical changes observed in this work. If a positive quantitative correlation were obtained between the time-course for development of cold-acclimation, the increase in the T_3/T_4 ratio in the tissues and the changes observed in this work on the one hand; and a positive quantitative correlation were obtained between the time-course for deacclimation, the decrease in the T_3/T_4 ratio in the tissues and the loss of the biochemical changes observed in this work on the other hand, then the evidence that thyroid hormones are a component of NST would be much stronger. At first glance, these correlations could be expected since Hillier (1968) showed that there was a positive correlation between the deiodination of T_4 and the urinary excretion of catecholamines in cold-exposed rats, the latter

already known to correlate positively with the development of cold-acclimation. (Leduc, 1961).

If the thyroid hormones were proven to be one of the components of NST in the sense that they are responsible for all or some of the biochemical changes described so far in the literature and in this thesis, then their relationship with the catecholamines (an essential component of NST) would require more study.

REFERENCES

Albright, E.C., Henninger, R.W., Larson. F.C (1965) in current topics in Thyroid Research (Eds. Cassano,C. and Andreoli L.) Acad. Press New York, 346 - 355.

Andersen, H.T., Christiansen, E.N., Grav, H.J., and Pedersen, J.I. (1970) Acta Physiol Scand., 80: 1 - 10.

Asano Y., Lieberman, V.A. and Edelman, I.S. (1976) J. Clin. Invest. 57: 368 - 379

Askew W.E., Huston, R.L. and Dohm, G.L. (1973) Metabolism (Clin, Exper.) 22: 473 - 480.

Babior, B.U. Greagan, S., Ingbar, S.H., and Kipnes, R.S. (1973) Nat. Acad. Sci. U.S.A. 70: 98 - 102.

Baldwin, K.M., Klinkerfuss, G.H., Terjung, R.L., Molé, P.A. and Holloszy, J.O. (1972). Am. J.Physiol. 222: 373 - 378

Balsam, A. (1974) J. Clin Invest 53: 980 - 987.

Balsam, A., and Leppo, L.E. (1974) Proc. Soc. Exper. Biol. Med. 145: 1401 - 1404.

Barker, S.B. (1964) in The Thyroid Gland, (ed. Pitt - Rivers, R., and Trotter, R.) Butterworths, London, 199 - 217

Barnard, R.J., and Peter, J.B. (1969)
J. Applied Physiol. 27: 691 - 695.

Barnard, T., Skala, J., and Lindberg, O. (1970)
Comp. Biochem. Physiol. 33: 499 - 528.

Bass, A., Brdiczka, D., Eyer, P., Hofer, S., and Pette, D. (1969)
European J. Biochem. 10: 198 - 206

Bass, A., Mackova, E., and Vitek., V. (1973)
Physiologia Bohemoslovaca. 22: 613 - 620

Behrens, W., and Himms-Hagen, J. (in press)
J. Bioenergetics.

Bernal, J., and Rey F. Escobar del. (1975 a)
Horm. Metab. Res. 7: 222 - 227

Bernal, J., and Rey F. Escobar del. (1975 b)
Acta Endocrinologica. 78: 481 - 492

Bernstein, J., Videla, L., and Israel, Y. (1975)
J. Pharmacol Exp. Ther. 192: 583 - 591.

Beyer, R.E. Noble, W.M., and Hirshfeld, T.J. (1961)
Fed. Proc. 20: 48

Beyer, R.E., Noble, W.M., and Hirshfeld, T.J. (1962)
Canad. J. Biochem. Physiol. 40: 511 - 518

Booth, F.W., and Holloszy, J.O. (1975)
Arch Biochem. Biophys. 167: 674 - 677

Borst, P. (1963) in Funktionelle und Morphologiske Organization.
der Zelle (Ed. Karlson, P.) New York, Springer-Verlag:
137 - 154

Brostrom, S. Fahlen, M Hjulmarson, A., and Johansson, R. (1974)
Acta, Physiol Scand. 90: 544 - 554

Brown - Grant, K. (1956)
J. Physiol. 131: 52 - 57

Bruni, A., Contessa, A.R., and Scalella, P. (1965)
Biochim. Biophys. Acta. 100: 1 - 12

Bricher, T., and Klingenberg, M. (1958)
Angenwandte Chemie. 70: 552 - 556

Buckowiecki, L.J., and Lindberg, O. (1974)
Biochim, Biophys. Acta. 348: 115 - 125

Bullock, G.R., Christian, R.A., Peters, R.F., and White, A.M. (1971) Biochem Pharmacol. 20: 943 - 953

Bullock, G.R. Carter, E.E., Elliott, P., Peters, R.F., Simpson, P., and White, A.M. (1972)
Biochem J. 127: 881 - 892

Bygrave, F.L., and Spencer, T. (1972)
Biochem. J. 129: 355 - 365

Callas. F., and Hayez, E. (1973)
Anat Res. 178: 539 - 544

Cannon, B. (1971)
European J. Biochem. 23: 125 - 135

Chen. J.C., Warshaw, J.B., and Sanadi, D.R. (1972)
J. Cell Physiol 80: 141 - 148

Christiansen, E.N., Pedersen, J.I., and Grav, H.J. (1969)
Nature 222: 857 - 860

Christiansen, E.N., Drahota, Z. Dusgynski, J., and Wojtzak , L.
(1973). European J. Biochem. 34: 506 - 512

Clark, M.G., Bloxham, D.P., Holland, P.C., and Lardy, H.A. (1973 a)
Biochem J. 134: 589 - 597

Clark, M.G., Williams, C.H., Pfeifer, W.F., Bloxham, D.P.,
Holland, P.C., Taylor, C.A., and Lardy. H.A. (1973 b)
Nature 245: 99 - 101

Cottle, M., and Carlson, L. (1956)
Endocrinology 59: 1 - 6

Cottle W.H. (1964)
Am. J. Physiol. 207: 1063 - 1066

D'Angelo, S. (1960)
Acta Endocrinol. 34(suppl. 51): 5 - 9

De Groot. E.M. (1968)
Am. J. Med . 44: 900 - 909

Dempsey, A.L., and Astwood, E.G. (1943)
Endocrinology. 32: 509 - 513

Depocas, F. (1958)

Canad. J. Biochem. Physiol. 36: 691 - 699

Depocas. F. (1960 a)

Fed. Proc. 19: 19 - 24

Depocas F. (1960 b)

Canad. J. Biochem. Physiol 38: 107 - 174

Depocas, F. (1960 c)

Fed. Proc. 19: 106 - 109

Depocas, F. (1962)

Am. J. Physiol 202: 1015 - 1018

Depocas. F. (1966)

Canad. J. Physiol. Pharmacol. 44: 875 - 880

Donnellan, J.F., Barker, M.D., Wood, J., and Beechy, R.B. (1970)

Biochem Biophys. Acta. 202: 35 - 39

Drabkin, D.L. (1950)

J. Biol. Chem. 182: 335 - 339

Eccles, J.C., Eccles, R.M., and Lundberg, A. (1958)

J. Physiol. 142: 275 - 291

Ershoff, B.H., and Colub, O.J. (1950)

Arch Biochem. 30: 202 - 206

Fain, J.N., and Jacobs, M.O. (1973)

Am. J. Physiol 224: 346 - 351

Faulkner, J.A., Maxwell, L.C., and Lieberman, D.A. (1971)

Am. J. Physiol. 221: 231 - 247

Fitts, R.H., Booth, F.W., Winder, W.W., and Holloszy J.O. (1975)

Am. J. Physiol. 228: 1029 - 1033

Flatmark, T., and Pedersen, J.I. (1975)

Biochem Biophys. Acta. 416: 53 - 103

Flattery K.V. and Sellers, E.A. (1972)

in Proc. Int. Symp. Environ. Bioenergetics. FASEB: 141 - 144

Foster, D.O. (1974)

Canad. J. Physiol. Pharmacol. 52: 1051 - 1062

Freinkel N and Lewis, D. (1957)

J. Physiol. 135: 288 - 300

Gale, C.C. (1973)

Ann. Rev. Physiol. 35: 391 - 430

Galton, V.A., and Nisula, B.C. (1969)
Endocrinology. 85: 79 - 83

Gardner, R.S. (1974)
Analytical Biochem. 59: 272 - 276

Gatehouse, P.W., Hopper. S., Schatz. L., and Segal, H.L. (1967)
J. Biol. Chem. 242: 2319 - 2324

Glock, G.E., and McLean, P. (1955)
Biochem. J. 61: 390 - 397

Glock, G.E., McLean, P., and Whitehead, J.K. (1956)
Biochem. J. 63: 520 - 524

Gollnick, P.D., and King, D.W. (1969 a) in Biochemistry of
Exercise, Medicine and S port: (ed. Poortmans, J.R.)
Basel, New York, 3: 239 - 244

Gollnick, P.D., and King, D.W. (1969 b)
Am. J. Physiol. 216: 1502 - 1509

Gollnick, P.D., Ianuzzo, C.D., and King, D.W. (1971)
in muscle Metabolism during exercise (Eds. Pernow,B.,
and Saltin, B.), Plenum Press, New York, London.

Gould, M.K., and Rawlinson, W.A. (1959)
Biochem. J. 73: 41 - 44

Gregerman R.I. (1963)
Endocrinology. 72: 382 - 386

Gross, N.J. (1971)
J. Cell Biol. 48: 29 - 40

Gustafsson, R., Tata, J.R., Lindberg, O., and Ernster, L. (1965)
J. Cell Biol. 26: 555 - 578

Hamilton, M.J., and Ferguson, J.H. (1972)
Comp. Biochem. Physiol. 43A: 815 - 824

Hannon, J.P. (1960)
Am. J. Physiol. 198: 740 - 744

Hansford, R.G., and Chappell, J.B. (1967)
Biochem. Biophys. Res. Commun. 27: 686 - 692

Harrison, T.S. (1964)
Physiol. Rev. 44: 161 - 185

Hart, J.S., and Jansky, L. (1963)
Canad. J. Biochem. Physiol. 41: 629 - 634

Haslam, J.M., and Krebs, D.E. (1968)
Biochem. J. 109: 921 - 925

Hayward, J.S., and Davies, P.F. (1972).
Canad. J. Physiol. Pharmacol. 50: 168 - 170

Hearn, G.R., and Wainio, W.W. (1956)
Am. J. Physiol. 185: 348 - 350

Heick, H.M.C., Vachon, C., Kallai, M.A., Bégin-Heick, N., and LeBlanc J. (1973).
Canad. J. Physiol. 51: 751 - 758

Hemon, P., and Berbey, B, (1968)
Biochim. Biophys. Acta. 170: 235 - 243

Heroux, O., and Petrovic, M.M. (1969)
Canad. J. Physiol Pharmacol. 47: 963 - 967

Hillier, A.P. (1968)
J. Physiol. 197: 135 - 147

Himms-Hagen, J. (1967)
Pharmacol. Rev. 19: 367 - 461.

Himms-Hagen, J. (1969)
J. Physiol. 205: 393 - 403

Himms-Hagen, J. (1970)
Adv. Enz. Reg. 8: 131 - 151

Himms-Hagen, J. (1971)
Canad. J. Physiol. Pharmacol. 49: 545 - 553

Himms-Hagen, J. (1972 a)
Lipids. 7: 310 - 323

Himms-Hagen, J. (1972 b) in Handbook of Experimental
Pharmacology (Eds. Blaschko, H., and Muscholl, E.)
Springer-Verlag, Berlin, New York. 33: 363 - 462

Himms-Hagen, J., Bukowiecki, L., Behrens, W., and Bonin, M. (1972)
in Proc. Int. Symp. Environ. Physiol. Bioenergetics. FASEB:
127 - 133

Himms-Hagen, J. (1975)
Handbook of Physiology Endocrinology VI. Adrenal Gland
(Eds. Blaschko, H., Sayers, G., and Smith, O.) 637 - 665

Himms-Hagen, J. Behrens, W., Muirhead, M., and Hbous, A. (1975)
Mol. Cell. Biochem. 6: 15 - 31

Himms-Hagen ,J. (1976)

Ann. Rev. Physiol (Ed. Knobil, E.) 38: 315 - 351

Hittelman, K.J., Lindberg, O., and Cannon, B. (1969)

European J. Biochem. 11: 183 - 192

Hogan, L.M., Dawson, D.M., and Romanul, F.C. (1965)

Arch. Neurol. 13: 274 - 282

Hohorst, H.J., and Rafael, J. (1968)

Zeitschrift fur Physiologische Chemie. 349: 268 - 270

Holloszy, J.O. (1967)

J. Biol. Chem. 242: 2278 - 2282

Holloszy, J.O., and Oscai, L.B. (1969)

Arch Biochem. Biophys. 130: 653 - 656

Holloszy, J.O., Oscai, L.B., Don, T.J., and Molé. P.A. (1970)

Biochem. Biophys. Res. Commun. 40: 1368 - 1373

Holloszy, J.O., Baldwin, K.M., and Winder, W.W. (1973)

Proc Soc. exp. Biol. Med. 143: 753 - 758

Horecker, E., and Kornberg, L. (1948)

J. Biol. Chem. 175: 325 - 330

Horwitz, B.A., Detrick, J.F., and Smith, R.Em. (1972)
Experientia. 28: 284 - 286.

Horwitz, B.A. (1975)

International Symposium on Depressed Metabolism and Cold Thermogenesis, Prague, 1974, Prague: Academia (in press).

Hsieh, A.C.L. (1962)
J. Physiol. 161: 175 - 188

Hsieh, A.C.L. (1963)
J. Physiol. London. 169: 851-861

Hsieh, A.C.L., and Carlson, L.D. (1957)
Am. J. Physiol. 190: 243 - 246

Hsieh, A.C.L., Pun, C.W., Li, K.M., and Ti, K.W. (1966)
Fed . Proc. 25: 1205 - 1209

Hue, L., and Hers, H.G. (1974)
Biochem. Biophys. Res. Commun. 58: 540 - 548

Ingbar, S.H., and Bass, D.E. (1957)
J. Endocrinology 15: ii - iii

Isaacs, G.H. Sacktor, B; and Murphy, T.A. (1969)
Biochem. Biophys. Acta. 177: 196-203

Ismail - Beigi, F., and Edelman, I.S. (1970)
Proc. Nat. Acad. Sci., U.S.A. 67: 1071 - 1078

Ismail - Beigi, F., and Edelman, I.S. (1971)
J. Gen. Physiol. 57: 710 - 722

Ismail - Beigi, F. and Edelman, I.S. (1974)
Proc. Soc. Exp. Biol. Med. 146: 983 - 988

Israel. Y., Videla, L., Mac Donald, A. Bernstein J. (1973)
Biochem. J. 134: 523 - 529

Jansky, L. (1966)
Fed. Proc. 25: 1297 - 1302

Jansky, L. (1971)
in "Nonshivering Thermogenesis" Prague Sympossium.
Academia: 159 - 172

Jansky,L. (1973)
Biol. Rev. 48: 85 - 132

Jansky, L., and Hart. J.S. (1963)

Canad. J. Biochem. Physiol. 41: 953 - 964

Jansky, L., and Hart, J.S. (1968)

Canad. J. Physiol. Pharmacol. 46: 653 - 659

Karmen, A. (1955)

J. Clin. Invest. 34: 131 - 133

Kendrick - Jones, J., and Perry, S.V. (1965)

Life Sciences 7: 957 - 960

Klain, G.J. (1963)

Biochim. Biophys. Acta. 74: 778 - 780

Klingenberg M (1967)

in methods in Enzymology (ed. Estabrook R.W., and Pullman, M.E.) X: 3 - 7

Klingenberg, M., and Pfaff. E. (1966)

BBA Library. 7: 180 - 184

Knigge, K.M. (1960)

Fed. Proc. 19(Suppl. 5) : 45 - 49

Kornacker M.S. and Ball, E.G. (1968)
J. Biol. Chem. 243: 1638 - 1644

Kraus, H., Kristen, R, and Wolff, J.R. (1969)
Pflu. Arch. European J. Physiol. 308: 57 - 79

Kubista, V., Kubistova, J. and Pette, D. (1971)
European J. Biochem. 18: 553 - 560

Kuner, J. M., and Beyer, R.E., (1970)
J. Membrane Biol. 2: 71 - 84

Lardy, H., Lee, Y.P., and Takemori. A.E. (1959)
J, Biol. Chem. 234 : 3051 - 3054

Laguens, R.P., and Gomez - Dumm, C.L. (1968)
Experientia (Basel). 24: 163 - 164

Leak, D.. (1970)
The Thyroid and the autonomic nervous system.
William Heinemann Medical Books Ltd., London.

Leblanc, J., and Villemaire, A. (1970)
Am. J. Physiol. 218: 1742 - 1745

Leblanc, J., and Pouliot, M (1964)
Am, J. Physiol. 207: 853 - 856

Leblanc, J., Lafrance, L., Villemaire, A., Roberge, C., Valliéres J., and Rousseau, S. (1972)
in Proc. Int. Symp. Environ. Physiol. Bioenergetics. FASEB: 71-76

Leblond, M., and Gross, L.A. (1943)
Endocrinology 33: 155 - 160

Leduc, J. (1961)
Acta Physiol. Scand. 53: Suppl. 183

Lee, Y.P., and Lardy, H.A. (1965)
J. Biol. Chem. 240: 1427 - 1436

Lehninger, A.L. (1974)
Circulation Res. 35: III 83 - 90

Lieberman. D.A., Maxwell, L.C., and Faulkner, J.A. (1972)
Am.J. Physiol. 222: 556 - 560

Lowry, O.H., Rosebrough, N.J., Tarr, A.L., and Randall, R.J.
(1951) J. Biol. Chem. 193: 265 - 275.

Marx, J.L. (1975)
Science. 189: 35 - 37

Matlib, M.A., and O'Brien, P.J. (1975)
Arch. Biochem. Biophys. 167: 193 - 202

Max, S.R. (1972)
Biochem. Biophys. Res. Commun. 46: 1394 - 1398

Max S.R. Garbus, J., and Wehman, E. (1972)
Analytical Biochem. 46: 576 - 584

Max, S.R., Rifenberick, D.H., and Gamble, J.G. (1973)
Am. J., Physiol 225: 1295 - 1299

Mejsnar. J., and Jansky, L. (1971)
Int. J. Biometeor. 15: 321 - 324

Molé, P.A. Oscar. L.B., and Holloszy J.O. (1971)
J. Clin. Invest. 50: 50 - 54

Munn, E.A., and Blair, P.V. (1967)
Zeitschrift für Zellforschung 80: 205 - 213

Muscatello, V. and Patriarca, P.L. (1968)
Am. J. Pathol 52: 1169 - 1189

Nachlas, M.M., Margulies S.I., and Seligman A.M. (1960)
J. Biol.Chem. 235: 499 - 503

Napolitano,L., and Fawcett, D. (1958)
J. Biophys. Biochem. Cytol. 4: 685 - 692

Neubert, D. Teske, S., Schmieder, M., Kohler, E., and
Oberdisse, E. (1970).
Arch. Pharmak. Exp. path. 262 : 264 - 294

Newsholme, E.A., and Underwood, A.H. (1966)
Biochem. J. 99: 24C

Nunn, R.E., (1970)
Electron microscopy: Preparation of Biological Specimens,
Butterworth.

Ohkawa, K - I., Vogt., M.T., and Farber, E. (1969)
J. Cell Biol. 41 : 441 - 449

Pedersen, J.I., and Grav, H.J. (1972)
European J. Biochem. 25: 75 - 83

Peter, J.B., Barnard, B.J., Edgerton, V.R., Gillespie C.A., and Stempel, K.E. (1972)
Biochemistry 11: 2627 - 2633

Pette D., Smith, M.E., Staudte, H.W., and Vrbova, G. (1973)
Pflugers Arch. 338: 257 - 272

Pitt _ Rivers, R., and tata. J.R. (1959)
The Thyroid Hormones. London: Perg mon press Ltd.

Purvis, J.L., and Lowenstein, J.M. (1961)
J. Biol. Chem. 236: 2794 - 2803

Rabinowitz, M., Aschenbrenner, V., Albin, R., Gross, N.J., Zak, R., and Nair, K.G. (1971)
In "Cardiac Hypertrophy" (ed. Alpert, N.R.). Acad. Press. New York and London : 283 - 299

Rabinowitz, M., and Zak, R. (1972)
Annual Rev.Med. 23: 245 - 262

Rand, C.C., Riggs, D.S., and Talbot, N.B. (1952)
Endocrinology. 51: 562 - 569

Reichlin, S., Bollinger, J., Nejad, I., and Sullivan, P. (1973)
Mount Sinai J. Med. 40: 502 - 510

Romanul, F.C., and Hogan, L.M. (1965)
Arch. Neurol. 13: 263 - 282

Romanul, F.C., and Van Der Meulen, J.P. (1966)
Nature. 212 : 1369 - 1370

Romanul, F.C., And Van Der Meulen, J.P. (1967)
Arch. Neurol. 17: 387 - 402

Rosengvist, V. (1972)
Acta Med. Scand. 192: 353 - 359

Sacktor, B., Bulos, B., Shukla, S. (1972)
Arch. Biochem. Biophys. 151 : 387 - 390

Safer, B., Smith, C.M., and Williamson, J.R. (1971)
J. Molecular and Cellular Cardiology. 2: 111 - 124

Schacterle, G.R., and Pollack, R.L. (1973)
Analytical Biochem. 51 : 654 - 655

Sellers, E.A., Flattery, K.V., and Steiner C. (1974)
Am. J. Physiol. 226: 290 - 294

Sellers, E.A., and You, S.S. (1950)
Am. J. Physiol. 163 : 81 - 85

Sellers, E.A., You, S.S., and Thomas, N. (1951)
Am. J. Physiol. 165: 481 - 485

Shertzer, E.N., and Cascarano, F.L. (1972)
Am. J. Physiol. 223: 632 - 636

Shrago, E., Lerner, E., Shug, A.L., and Elson, C. (1972)
J. Biol. Chem. 247: 1513 - 1519

Shum, A., Johnson, G.E., and Flattery, K.V. (1969)
Am. J. Physiol. 216: 1164 - 1169

Singer, T.P., and Lusty, C.J., (1960)
Biochem. Biophys. Res. Commun. 2: 276 - 281

Skaane, O., Christiansen, E.N., Pedersen, J.I., and Grav, H.J. (1972)
Comp. Biochem. Physiol. 42 B : 91 - 107

Slater, E.M., and Miledi, L. (1968)
J. Cell Sci. 3: 49 - 54

Smith, R.E. (1960)
Fed. Proc. 19 (Suppl. 5) : 146 - 151

Smith, R.E. and Hoijer, D.J. (1962)
Physiol. Rev. 42 : 60 - 142.

Smith, R.E. (1964)

Nature. 204: 1311 - 1315.

Sokoloff, L., and Kaufman, S. (1959)

Science 129: 569 - 573

Sottocasa, G.L., Kuylenstierna, B. Ernster, L., and Bergstrand, A. (1967)

J. Cell Biol. 32 : 415 - 419

Spiro, M.J., and Ball, E.J. (1958)

J. Biol. Chem. 231: 31 - 40

Staudte, H.W., Exner, G.V., and Pette, D. (1973)

Pflugers Arch. European J. Physiol. 344 : 159 - 168

Stevens, E.D., and Kido, M (1974)

Comp. Biochem. Physiol. 47 A: 395 - 397

Stevens, C.E., D'Angelo, S.E., Pashkis, K.E., Cantarow, A., and Sunderman, R.W. (1955)

Endocrinology. 56: 143 - 147

Suko, J. (1971)

Biochim. Biophys. Acta. 252: 324 - 327

Syrovy, E.M., Gutmann. E., and Melichna, J. (1972)
Physiol. Bohemoslov. 21 : 633 - 638

Tata, J.R., Ernster, L., Lindberg, O., Arrhenius, E., Pedersen, S. and Hedman, R. (1963)
Biochem J. 86: 408 - 428

Tauchi, H., Yoshioka, T., and Kobayashi, H. (1971)
Gerontologia. 17: 219 - 227

Tomanek, R.J., and Karlson, U.L. (1973)
J. Ultrastructural Res. 42: 201 -220

Van Linge, B. (1962)
J. Bone Jt. Surg. 44 : 711 - 721

Varis, R., Leppaluoto, J., Ranta, T., and Lybeck, H. (1974)
Acta Physiol. Scand. 90 : 640 - 644

Veldstra, H. (1956)
Pharmacol Rev. 8: 339 - 387

Venable, J.H., and Coggeshall, R. (1965)
J, Cell Biol. 25: 407 - 408

Videla, L., Flattery, K.V., Sellers, E.A., and Israel, Y (1975)
J. Pharmacol Exp. Ther. 192: 575 -582.

Waldstein, S.S. (1966)
Ann. Rev. Med. 17 : 123 - 152

Wallach, S., Bellavia, J.V., Gamponia. P.G., and Bristrim, P (1972) J. Clin. Invest. 51: 1572 - 1577

Weber, V.E. (1972)
J. Cell Biol. 55: 457 - 470

Williamson, J.R., Browning, E.T., Scholz, R. Kreisberg, R.A., and Fritz, I.B. (1968)
Diabetes 17: 194 - 208

Williamson, J.R., Jacob, A., and Scholz. R (1971)
Metabolism. 20: 13 - 26

Wilson, D.F., Stubbs, M., Veech, R.L., Erecinska, M., and Krebs, H.A. (1974)
Biochem. J. 140: 57 - 64

Winder, W.W.., Baldwin, K.M., Terjung, R.L., and Holloszy, J.O. (1975)
Am. J. Physiol. 220: 1341 - 1345.

Winkler, H.H., Bygrave, F.L., and Lehninger, A. (1968)
J. Biol. Chem. 243: 20 - 28

Wroblewski, L. and La Due, J.S. (1955)
Ann. Internal Med. 43: 345 - 360

Yamada, M. (1973)
Am. J. Physiol. 224: 1420 - 1424

Yamada, T., Fukuda, H., Takemura, Y., and Schichno, K. (1969)
Metabolism. 18 : 339 - 347

Young, H.L., and Pace, N. (1958)
Arch. Biochem. Biophys. 76: 112 - 121

Zaimis, E., Papadaki, L. Ash, A.S.F., Larbi, E., Kakari, S., Matthew, M., and Paradelis, A. (1969)
Cardiovascular res. 3 : 118 - 133.