MITOCHONDRIAL PROTEIN SYNTHESIS

AND

COLD-ACCLIMATION

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ABBREVIATIONS

AA	-	amino acid
ADP	-	adenosine-5'-diphosphate
ATP	-	adenosine-5'-triphosphate
BAT	-	brown adipose tissue
CA	-	cold-acclimated
CE	-	cold-exposed
EDTA	-	ethylene diamine tetraacetic acid
IBAT	-	interscapular brown adipose tissue
NA	-	noradrenaline
NST	-	nonshivering thermogenesis
M-DNA	-	mitochondrial DNA
SNS	-	sympathetic nervous system
tRNA	-	transfer RNA
TRIS	-	tris-hydroxymethyl-aminomethane
WA	-	warm-acclimated

TABLE OF CONTENTS

I. PURPOSE OF THE THESIS 1

II. LITERATURE REVIEW 2

A. ACCLIMATION TO COLD AND NONSHIVERING THERMOGENESIS... 2

1. Introduction 2
2. The physiological classification of thermogenic processes: shivering and nonshivering thermogenesis 3
3. Involvement of the sympathetic nervous system in acclimation to cold and nonshivering thermogenesis 7
4. Relative participation of various organs in nonshivering thermogenesis 22
5. Role of brown adipose tissue in nonshivering thermogenesis 29
6. Biochemical classification of thermogenic processes 35

B. THE BIOGENESIS OF MITOCHONDRIA 39

1. Introduction 39
2. Mitochondrial DNA and its coding capacity....... 41
3. Mitochondrial DNA polymerase 43
4. Mitoribosomes 43
5. Mitochondrial RNA's and mitochondrial RNA polymerase 44
6. Protein synthesis by isolated mitochondria as related to the physiological state of various tissues 46

7. The localization of mitochondrial enzymes....... 50

8. The search for the proteins made by the mitochondrion.................................... 53

9. Quantitative estimations of mitochondrial protein synthesis "in vivo"..................... 61

10. The relation between mitochondrial protein synthesis and mitochondrial function............ 61

11. The mechanisms of mitochondrial biogenesis...... 67

C. THE RELATION BETWEEN ACCLIMATION TO COLD AND THE BIOGENESIS OF MITOCHONDRIA........................... 79

III. MATERIAL AND METHODS................................. 84

A. MATERIALS.. 84

1. Rats.. 84

2. Chemicals... 84

B. METHODS.. 85

1. Removal of the organs and determination of their wet weight... 85

2. Injection of (^{14}C)-leucine.......................... 85

3. Isolation of the mitochondria..................... 85

4. Fractionation of the mitochondria................. 87

5. Preparation of the proteins for the counting of radioactivity...................................... 89

6. Protein estimation................................ 90

7. Counting procedures............................... 90

8. Amino acid incorporation by isolated mitochondria.. 90

9. Estimation of leucine concentration in various tissues of the rat.................................... 92

10. Methods of calculation and statistical evaluation of the data..................................... 93

IV. RESULTS.. 94

1. Determination of the half-lives of different mitochondrial protein fractions of liver, kidney interscapular brown adipose tissue and skeletal muscle of warm- and cold-acclimated rats........ 94

a) Purpose of the experiment.................... 94

b) Principle of the technique.................. 94

c) Description of the experiment................ 95

d) Results.................................... 95

e) Discussion.................................. 99

2. Determination of the "in vivo" rate of amino acid incorporation into various mitochondrial protein fractions of liver, brown adipose tissue and skeletal muscle of warm-acclimated, cold-acclimated and cold-exposed rats................ 112

a) Purpose of the experiment.................... 112

b) Principle of the technique.................. 112

c) Description of the experiment................ 113

d) Results.................................... 114

e) Discussion.................................. 124

3. Mitochondrial protein synthesis in isolated mitochondria of liver, brown adipose tissue and skeletal muscle of warm-acclimated, cold-acclimated and cold-exposed rats.................. 125

a) Purpose of the experiment.................... 125

b) Principle of the technique.................. 126

c) Description of the experiment................ 128

d) Results.................................... 129

e) Discussion.................................. 135

4. Determination of leucine concentration in various tissues of the warm-acclimated, cold-acclimated and cold-exposed rats.................. 140

a) Purpose of the experiment...................... 140

b) Principle of the technique..................... 142

c) Description of the experiment.................. 142

d) Results.. 143

e) Discussion..................................... 146

V. GENERAL DISCUSSION AND CONCLUSION........................ 148

VI. REFERENCES... 155

VII. APPENDIX... 170

LIST OF FIGURES

Figure 1: Rapid increase in shivering followed by a slow decrease during acclimation to cold......... 6

Figure 2: Effects of exposure to cold on excretion of norepinephrine, normetanephrine and 3-methoxy-4-hydroxyphenylglycol.................................... 9

Figure 3: Increased capacity for nonshivering thermogenesis in cold-acclimated rats......................... 13

Figure 4: Oxygen consumption and rectal temperature of curarized cold-acclimated rats and warm-acclimated rats.. 15

Figure 5: Enhancement of the calorigenic response to noredrenaline by cold-acclimation..................... 17

Figure 6A: Development of the enhanced calorigenic response to noradrenaline during acclimation to cold...... 19

Figure 6B: Decrease of the calorigenic response to noradrenaline in cold-acclimated rats at 5°C exposed to 25°C.. 19

Figure 7: Effect of the acclimation temperature on the metabolic response due to intramuscular injections of noradrenaline or adrenaline in anesthetized rats, kept at different temperatures for 3 weeks prior to experiments........................... 20

Figure 8: Persistence of nonshivering thermogenesis in functionally eviscerated rats....................... 26

Figure 9: Effect of removal and replacement of the interscapular brown adipose tissue of warm- and cold-acclimated rats on the calorigenic response to noradrenaline.................................. 32

Figure 10: Effect of prior removal of interscapular brown adipose tissue on the development of the enhanced calorigenic response to noradrenaline during acclimation to cold............................ 34

Figure 11: Regression lines of various mitochondrial protein fractions of liver, kidney, brown adipose tissue and skeletal muscle of warm- and cold-acclimated rats.. 100

Figure 12: Half-lives of various mitochondrial protein fractions of liver, kidney, brown adipose tissue and skeletal muscle of warm-and cold-acclimated rats.. 107

Figure 13: 14 15 Specific activities of various mitochondrial protein fractions of liver(Fig. 13). brown adipose tissue (Fig. 14) and skeletal muscle (Fig. 15) in warm-acclimated, cold-acclimated and cold-exposed rats killed 5 minutes after the intravenous injection of ^{14}C-leucine........................ 115

Figure 16; 17 18 Specific activities of various mitochondrial protein fractions of liver (Fig. 16), brown adipose tissue (Fig. 17) and skeletal muscle (Fig.18) in cold-exposed or cold-acclimated rats killed 5 minutes after the intravenous injection of ^{14}C-leucine. The specific activities were calculated as percentage of the corresponding control warm-acclimated groups........................ 119

Figure 19: Specific activities of isolated liver mitochondria, incubated at various concentrations of ATP 130

Figure 20: 21 Specific activities of isolated brown adipose tissue mitochondria (Fig. 20) and skeletal muscle (Fig. 21) incubated at various concentrations of ATP.. 132

Figure 22: A comparison of brown adipose tissue changes in mitochondrial QO_2, mitochondrial "in vivo" and "in vitro" amino acid incorporation during acclimation to cold with corresponding variations observed during the development of Neurospora crassa cells.. 138

Figure 23: Leucine concentrations in plasma, liver, interscapular brown adipose tissue and skeletal muscle of warm-acclimated, cold-acclimated and cold-exposed rats................................ 144

ABSTRACT

Major changes in the mitochondrial protein synthesizing system of brown adipose tissue and skeletal muscle mitochondria, but not liver mitochondria, have been shown to be associated with the development of nonshivering thermogenesis during acclimation to cold of the rat. Three different experimental approaches were used:

- the determination of the half-life of mitochondrial proteins and of various mitochondrial protein fractions in the intact animal;
- the measure of the "in vivo" ^{14}C-leucine incorporation in mitochondrial proteins and various mitochondrial protein fractions together with the determination of "free" leucine concentration in plasma and various tissues;
- the determination of the "in vitro" amino acid incorporation in mitochondrial proteins.

It is postulated that alterations in metabolism of certain proteins synthesized by brown adipose tissue and skeletal muscle mitochondria are directly or indirectly responsible for the development and for the maintenance of the increased capacity for making heat by nonshivering thermogenesis that characterize the cold-acclimated state.

I. PURPOSE OF THE THESIS

The main purpose of this thesis is to evaluate the possible role of the mitochondrial protein synthesizing system in the regulation of the mechanisms of heat production in various tissues of the rat under a physiological condition which is known to produce important qualitative and quantitative changes in the basic mechanisms of energy regulation, namely cold-acclimation.

The consequent objective is to study the relations between mitochondrial protein synthesis and

a) the mechanisms of nonshivering thermogenesis (NST)

b) the mechanisms responsible for the development of the increased capacity for NST

during cold-exposure and cold-acclimation of the rat.

The principal question asked is: "Is the mitochondrial protein synthesizing system directly involved in and responsible for the development of NST during acclimation to cold of the rat"?

As this thesis involves the study of a correlation between the biogenesis of mitochondria and acclimation to cold, the literature review which follows is divided accordingly in three parts:

A. Acclimation to cold and nonshivering thermogenesis

B. The biogenesis of mitochondria

C. The relation between acclimation to cold and the biogenesis of mitochondria

II. LITERATURE REVIEW

A. ACCLIMATION TO COLD AND NONSHIVERING THERMOGENESIS

1. INTRODUCTION

It is essential for homeotherms exposed to a cold environment to maintain their body temperature constant and to adapt in a very rapid and efficient way to this stress. It is therefore not surprising that they have developed several different mechanisms to increase their heat production or to decrease their heat loss. Carlson and Hsieh (1970) have derived a simple equation describing heat exchange between an organism and its environment:

$$Tb = I(M - E) + Ta$$

where Tb = body temperature (central temperature)

Ta = a composite temperature of the environment

I = insulation factor

M = heat generated by metabolic reactions

E = evaporative heat loss

This equation means that in order to maintain its body temperature (Tb) constant, the animal has at its disposal means of alterating its insulation (I), its metabolic rate (M) or its evaporative heat loss (E). Heat loss is in general prevented by "physical" means (i.e. changes in I or E) such as the laying-down of subcutaneous fat, piloerection, peripheral vasoconstriction and heat production is elevated by an increase in the meta-

bolic rate of the animal (M). The rat responds to cold-exposure by altering I,E and M, but the marked and sudden increase in its metabolic rate is quantitatively the essential mechanism which contributes to a constant body temperature.

2. THE PHYSIOLOGICAL CLASSIFICATION OF THERMOGENIC PROCESSES: SHIVERING AND NONSHIVERING THERMOGENESIS

For many years physiologists have classified mechanisms of heat production in two categories: shivering thermogenesis and non-shivering thermogenesis (NST).

- Shivering thermogenesis

The mechanism of the muscle-localized shivering thermogenesis is relatively well-understood. The ATPase associated with shivering produces an increased supply of ADP to the mitochondrion. ADP rather than Pi stimulates the oxygen consumption of the tightly coupled muscle mitochondria and its own rephosphorylation into ATP (Chance and Williams 1955, a,b,c). Increased quantities of heat are produced during the breakdown of ATP and during the stimulated respiration of the mitochondrion.

The link between increased shivering and increased heat production is demonstrated by "in vivo" experiments in which the suppression of shivering by curare results in an inhibition of the increased metabolic rate which normally parallels shivering. The curarized warm-acclimated rat (WA) exposed to cold becomes very quickly hypothermic and dies (Cottle and Carlson 1956). Thus,

the first and essential stimulus of shivering thermogenesis is the release of acetylcholine by motor nerves.

- Nonshivering thermogenesis

The mechanism of NST is not understood. By definition and, as its name implies, NST represents any thermogenesis which is not mediated by shivering. This is not a very precise definition because "a priori" it could be localized not only in muscle, but also in other different tissues and therefore be mediated through different mechanisms.

The rat and many other species are able to use the shivering and the NST mechanisms in response to a cold stress in order to increase their heat production, but the extent to which they will use only one of them or certain proportions of both mechanisms will depend on such factors as their age, the magnitude of the cold stress, the time of cold-exposure, if they are hibernating animals or not.

The new-born rat, for example, does not shiver when exposed to cold and maintains its body temperature constant. It is thus producing heat by NST, but during its growth at normal temperature it progressively loses the capacity for NST, so that the adult, WA rat will respond mainly to a cold stress by shivering and not by NST. However, if that rat is maintained in the cold (5°C) for a period of 4-6 weeks, shivering as measured by electromyogram progressively disappears without any major change

in the elevated metabolic rate (Hart et al. 1956), (Fig. 1). Thus, the adult rat is able to regain the capacity for making NST it had just after birth only by a prolonged stay in the cold.

Any interpretation or measurement at the molecular level of a phenomenon related to NST should account for these fundamental characteristics: (1) NST is a facultative process. To quote Depocas (1960):

> "Nonshivering thermogenesis should not be a permanent manifestation of a tissue but be subjected to being turned on and off, just like shivering in the warm-acclimated rat, in response to changes in environmental temperature".

NST is thus turned off in isolated tissues or cells, in homogenates, in isolated mitochondria etc., just as shivering thermogenesis is not present in an isolated piece of muscle. NST is present only "in vivo" in certain tissues of the rat when it is exposed to a cold stress. (2) NST is an adaptative process. To quote Himms-Hagen(1970):

> "NST has been demonstrated to occur only in certain new-born species (rabbit, rat, guinea pig, human) in hibernating mammal (bat, hamster, ground squirrel) and in certain species that have been acclimated to cold (rat, rabbit). It does not occur when they are adult, when they are not hibernating or when they are not acclimated to cold".

Thus, the capacity for making NST could be, at least during a certain period of time, a definite characteristic of certain tissues directly or indirectly involved in making or controlling NST.

From these considerations it is clear that the study of adaptation of the rat to cold is a very useful experimental problem

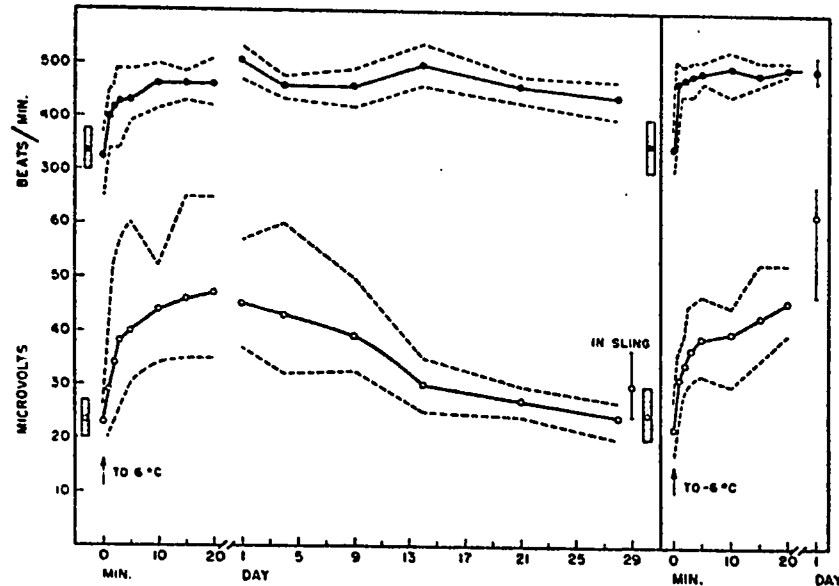


Figure 1: Rapid increase in shivering followed by a slow decrease during acclimation to cold.

The lower part of the diagram shows muscle electrical activity and the upper part, the heart rate in function of the time of exposure at 6°C. After 30 days the cold-acclimated rats were moved from +6°C to -6°C (right-hand part of the diagram). The broken lines show the range of variation and the cross-hatched vertical bars show the muscle electrical activity or the heart rate in warm-acclimated rats living at 30°C.

(From Hart et al. 1956).

for the understanding of the two basic phenomena characterizing NST: the relatively slow mechanism controlling the development of the capacity for making it and the very rapid mechanism which switches it on and off.

3. INVOLVEMENT OF THE SYMPATHETIC NERVOUS SYSTEM IN ACCLIMATION TO COLD AND NONSHIVERING THERMOGENESIS

The sympathetic nervous system (SNS) is activated during the cold-exposure of warm-acclimated (WA) and cold-acclimated(CA) rats and there is good evidence that noradrenaline(NA) liberated from the sympathetic nerve endings rather than adrenaline mediates the switching on and off of NST. NA is also the principal hormone involved in the development of the capacity for making NST. Evidence for this has been reviewed by Himms-Hagen (1967; 1972, a) and will only be briefly summarized here according to three different types of experimental approaches: (1) the measure, (2) the inhibition and (3) the mimicking of the activity of the SNS during cold-exposure and cold-acclimation. It should be added that the SNS is not only deeply involved in the development of NST and in the mechanism of switching on and off heat production, but is also the main regulator of the necessary substrate mobilization without which no extra heat could be produced.

(1) Methods which measure the activity of the sympathetic nervous system: the measure of the synthesis and excretion of catecholamines or related metabolites.

Increased synthesis, excretion and turnover of catecholamines

is generally considered a good indication that the SNS is activated (Himms-Hagen 1967). There is an increased urinary excretion of adrenaline and NA and various other related metabolites in WA and CA rats exposed to cold (Leblanc and Nadeau 1961; Leduc 1961; Sellers et al. 1972). These studies have shown that:

a) The excretion of catecholamines is proportional to the temperature of exposure and is greater in younger animals (Leduc 1961).

b) The excretion of NA rises five to six times in the first days of cold exposure, reaching its peak at about one week. Thereafter its excretion declines but remains at elevated levels during several weeks (Leduc 1961; Sellers et al. 1972).

c) The pattern of adrenaline excretion is different; after an initial increase with a maximum at 7 days, the excretion returns to normal levels when the rat is acclimated to cold (Leduc 1961).

d) If the CA rat which excretes and synthesizes much more NA than the WA rat is returned to the warm, the excretion values drop to normal within one or two days (Leduc 1961).

e) In addition to an increased excretion of catecholamines there is also during acclimation to cold increased excretion of various metabolites of the catecholamines (Fig. 2).

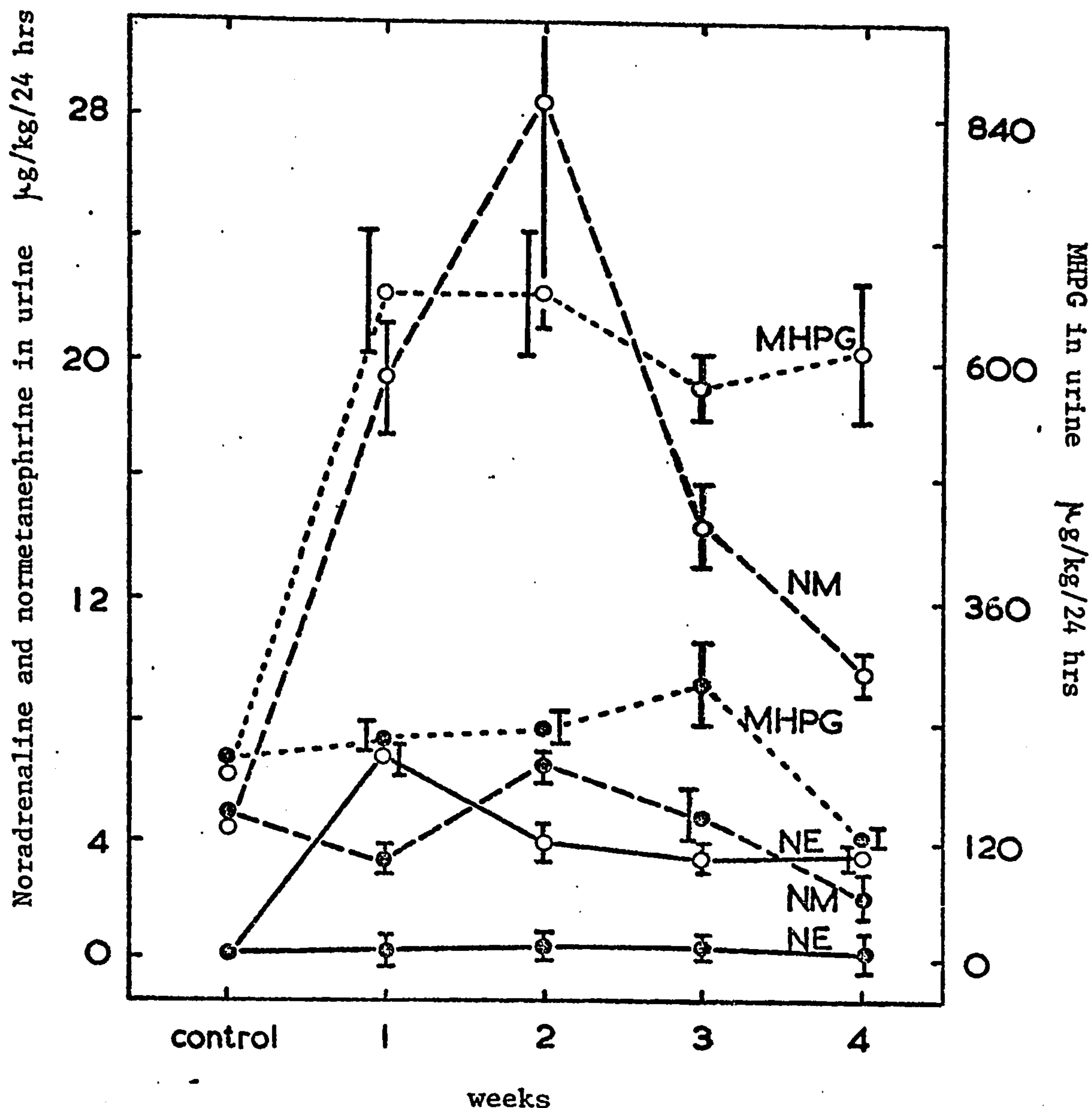


Figure 2:

Effects of exposure to cold ($4^{\circ}C$) on excretion of norepinephrine (NE), normetanephrine (NM), and 3-methoxy-4-hydroxyphenylglycol (MHPG). Open circles represent rats exposed to $4^{\circ}C$. Filled circles represent rats kept at $30^{\circ}C$. Points represent means or 4 values. Standard errors are plotted for weeks 1, 2, 3 and 4.

(From Sellers et al. 1971).

The 3-methoxy-4-hydroxy-phenylglycol (MHPG) is the major metabolite of adrenaline and NA in the rat. Its excretion rises sharply during the first hours of cold exposure and after a period of fluctuations remains constant at elevated levels during acclimation to cold (Sellers et al. 1972).

The main conclusions of the studies of catecholamine excretion is that they are very useful as an index of activation of the SNS, but they cannot measure quantitatively that activation, because of such problems as the fact that much of the NA secreted from the nerve endings is taken up again in the same nerve endings and will therefore never appear in the urine in any form (Himms-Hagen 1972,a). The many problems of quantitative evaluation of the increased synthesis, excretion and turnover of catecholamines during acclimation and exposure to cold have been discussed by Himms-Hagen (1972,a). The main conclusions are that NA and adrenaline synthesis and excretion may proceed at rates independent one of another, that they are both activated on cold exposure, that their excretion declines after one week, and that NA, but not adrenaline, synthesis and excretion remains elevated during all the time the rat stays in the cold.

(2) Methods which reduce the activity of the sympathetic nervous system.

The rationale of this experimental approach is: if the SNS is directly involved in adaptation to cold, methods which artificially inhibit or reduce its activity should also inhibit the

the ability of the rat to survive and to adapt to cold.

It is possible to inhibit the activity of the SNS by immunological, surgical (adrenalectomy, adrenodemedullation, sympathectomy) or by pharmacological means (the use of ganglion blocking agents, adrenergic blocking agents, drugs which prevent the release of NA from nerve endings, drugs which inhibit the synthesis of catecholamines, etc.).

In general, the single use of <u>one</u> of those methods, like adrenodemedullation, impairs, but does not inhibit totally the ability of the rat to maintain its body temperature constant and to survive in the cold. More drastic procedures have to be used, like a combination of immunosympathectomy and adrenodemedullation, or adrenodemedullation and inhibition of catecholamine synthesis (inhibition of dopa decarboxylase), to show that the activity of the SNS is essential for the survival in the cold. However, older rats seem to be less dependent on the activity of the SNS than younger ones as shown by the fact that the older rats survive in the cold even when immunosympathectomized and adrenodemedullated (Schönbaum 1966). Young rats equally treated become very quickly hypothermic and die (Berti et al. 1965). It is surprising that in the older rats the excretion of NA is similar to that of the controls and is probably due to a remnant activity of the SNS.

Moreover, those methods do not distinguish between the contribu-

tion of the shivering or nonshivering mechanism that the rat is using for increasing its heat production when a part of its SNS is inhibited. The differentiation between the two mechanisms of thermogenesis was achieved in a series of elegant experiments performed by Cottle and Carlson (1956), in which shivering was inhibited by curare. Figure 3 shows that curarized WA rats exposed to cold become very quickly hypothermic after a small initial increase in their metabolic rate, whereas curarized CA rats have a normal increase in their metabolic rate, which permits them to maintain their body temperature during at least 2 hours. This proves that the WA animals have a small or no capacity for making NST, whereas the CA animals can rely on that sole mechanism for maintaining their body temperature. Cottle and Carlson (1956) also investigated the role of adrenal medulla in NST by the surgical removal of that organ 10 days before the experiment in CA and WA animals. They proved that the presence of the adrenal medulla was not essential for the maintenance of a normal metabolic rate of both groups of animals at room-temperature, but that the lack of that organ impairs but does not suppress completely the marked increase in metabolic rate observed in the CA group, suggesting that the adrenal medulla takes part in NST, but is not essential for the process.

A similar experiment was performed by Hsieh and Carlson (1957) with the use of a much more drastic technique than adrenodemedullation: the use of the ganglion blocking agent hexamethonium,

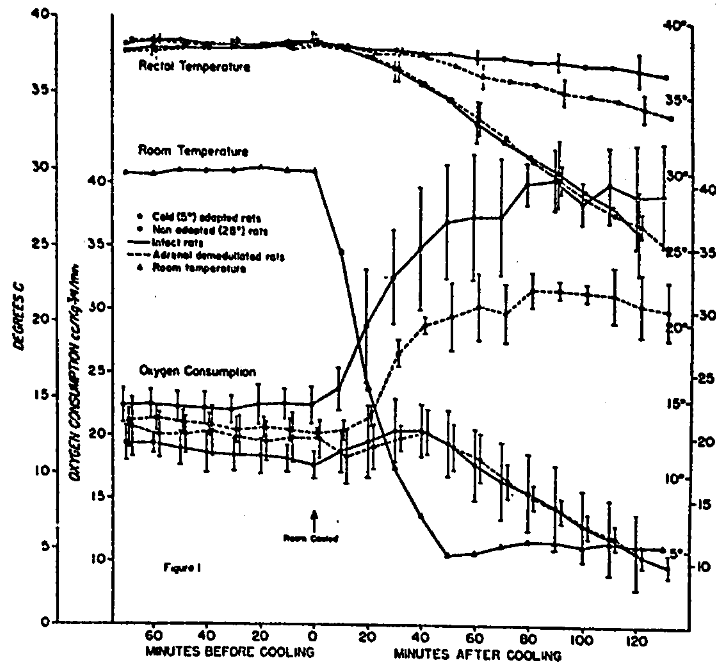


Figure 3: Increased capacity for NST in CA rats.

Metabolic response of curarized WA and CA rats to lowered ambient temperature, and the effect of adrenal demedullation on NST are shown. Mean oxygen consumption, rectal temperature and ambient temperature are plotted on ordinate; minutes before and after cooling on abscissa.

(From Cottle and Carlson 1956).

which, by inhibiting transmission in sympathetic ganglia, prevents the release of NA from nerve endings and of adrenaline and NA from adrenal medulla. They showed that if hexamethonium is administered to curarized CA rats before cold-exposure, the increase in their metabolic rate is totally inhibited (adrenodemedullation in the experiment of Cottle and Carlson inhibited it only partially), and if administered after cold-exposure, when oxygen uptake is high, it decreases markedly the elevated metabolic rate (Fig. 4). NA, but not adrenaline, prevents this decrease in oxygen consumption, suggesting at first sight that NA is more effective than adrenaline in curarized CA rats.

However, the catecholamines were administered by intramuscular injection and it is not possible to draw any definitive conclusion regarding the efficiency of those hormones to reverse hexamethonium effects, because it is possible that adrenaline is more slowly absorbed than NA by this kind of injection (Himms-Hagen 1972, a). In fact, adrenaline and NA administered by intravenous injection are equally efficient in the mimicking of NST (Himms-Hagen 1972, a).

In summary, these methods have demonstrated that the SNS is directly involved in NST, but that it is very difficult to inhibit it entirely, probably because the SNS is a highly integrated and adaptable mechanism as both parts of its two major divisions (the adrenal medulla and the sympathetic nerve endings) can take over the functions of the other part when necessary (Himms-Hagen 1967).

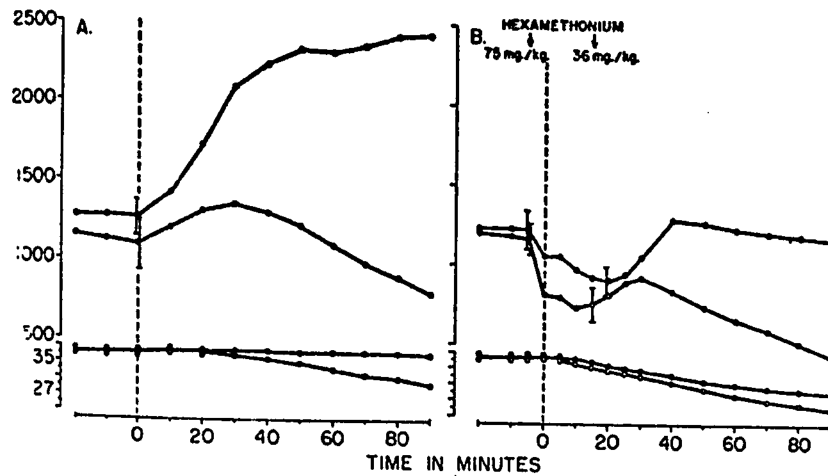


Figure 4:

Oxygen consumption and rectal temperature of curarized CA rats (•) and WA rats (o). The room-temperature was cooled to 5°C at zero time. Hexamethonium was injected intravenously at the points indicated by arrows.

(From Hsieh and Carlson 1957).

(3) Methods which mimick the activity of the sympathetic nervous system: the calorigenic action of adrenaline and noradrenaline.

The CA rat which, excretes and synthesizes much more NA than the WA rat, has also an increased capacity to respond to the catecholamines. The marked difference between the response of a WA and a CA animal to the intravenous infusion of NA is shown in Fig.5. Intravenous infusion of adrenaline induces similar responses. This enhancement in the calorigenic response is not due to an increase in sensitivity to NA, but to an increase of the maximum attainable response (Himms-Hagen 1972, b). In fact, the increase in the metabolic rate of the CA rat due to intravenous infusion of NA approaches the maximum capacity of the rat for oxygen consumption and is of the same magnitude as its metabolic rate in the cold (Jansky 1966).

Actually the NA calorigenic effect is directly related to the development of NST during acclimation to cold as shown by the following experiments:

a) A comparison of Fig. 1 and 6A shows that when a rat is exposed to cold, shivering, as measured by electromyogram, increases in about 20 minutes to a maximum, and, if the rat is maintained in the cold, decreases very slowly during 4 weeks. This means that NST (which is total thermogenesis minus shivering thermogenesis) changes in an inversely proportional way. During that same period there is a very

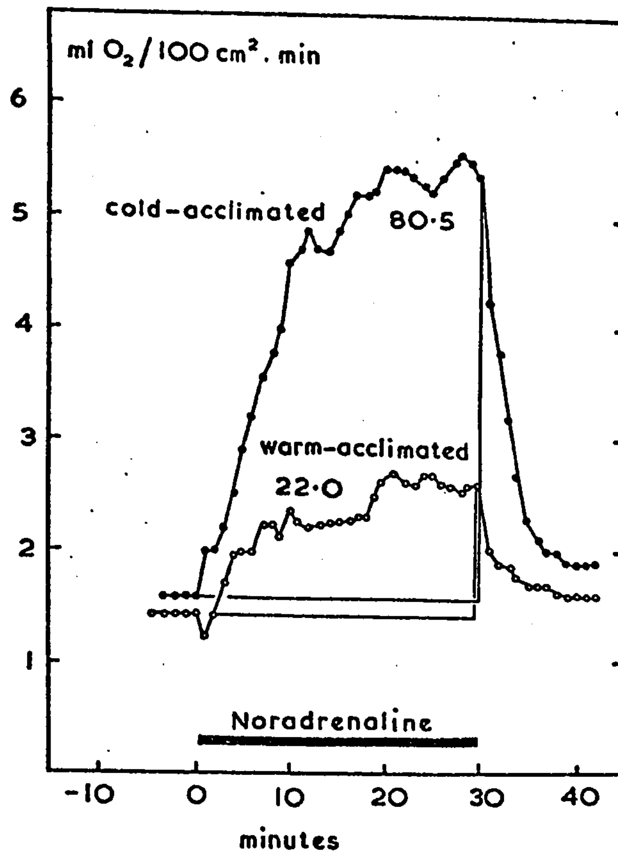


Figure 5: Enhancement of the calorigenic response to noradrenaline by cold-acclimation. Oxygen uptake of a warm- and a cold-acclimated rat during infusion of noradrenaline is shown. Noradrenaline was infused intravenously, 0.5 microg/100cm^2. minute, from 0 to 30 minutes. The WA rat had lived at room temperature (25°-28°C) and the CA rat in the cold (4°C) for 13 weeks. The values of 80.5 and 22.0 on the graph are obtained from the area under the curve during the 30 minutes of infusion of noradrenaline and they represent the total increase in oxygen uptake in ml O_2/100 cm^2. in 30 minutes.

(From Himms-Hagen 1970).

slow progressive increase in the capacity to respond to intramuscular injection of NA, which parallels the development of NST. The maximum calorigenic effect of NA is attained after 4 weeks, when shivering has totally disappeared (Fig. 6A). Intravenous infusion of NA gives similar results (Himms-Hagen 1969; Depocas 1960).

b) The magnitude of the calorigenic action of NA progressively disappears during cold-deacclimation (or deadaptation of CA rats from cold) (Fig. 6B).

c) The magnitude of the calorigenic action of NA is inversely proportional to the acclimation temperature (Fig. 7) (Jansky et al. 1967).

d) Another characteristic is that the speed of the development of the increased capacity to respond to NA is independent of different acclimation temperatures ranging from +15°C to -1°C (Bartunkova et al. 1971) (Fig. 6A). It is surprising that it takes as much time for a rat to adapt to a relatively mild cold-exposure of +15°C as to adapt to -1°C. The intensity of the cold stress seems to determine only the magnitude of the response to NA (or the magnitude of NST) during and after acclimation, but not the speed of that acclimation (or the speed of the development of NST). This suggests that the rate limiting steps of the development

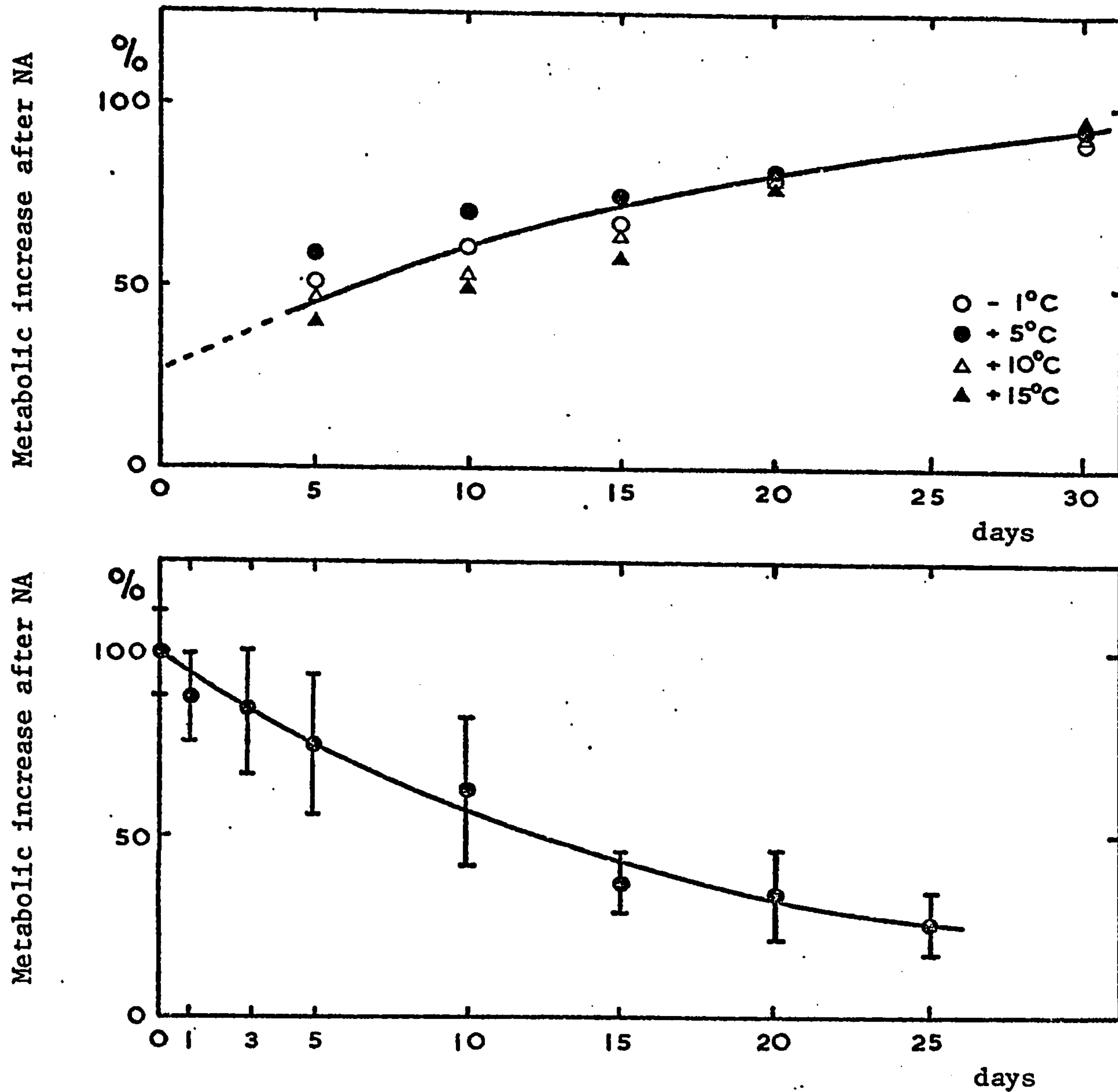


Figure 6A: Development of the enhanced calorigenic response to NA during acclimation to cold. Metabolic response to intramuscular injections of NA (0.4 mg of NA/Kg) are expressed as percentage of the value obtained after 40 days of acclimation to different temperatures.

Figure 6B: Decrease of the calorigenic response to NA in CA rats at 5°C exposed to 25°C . Metabolic response to intramuscular injections of NA (0.2 mg NA/Kg) are expressed as percentage of the initial metabolic response of CA rats.
(From Bartunkova et al. 1971).

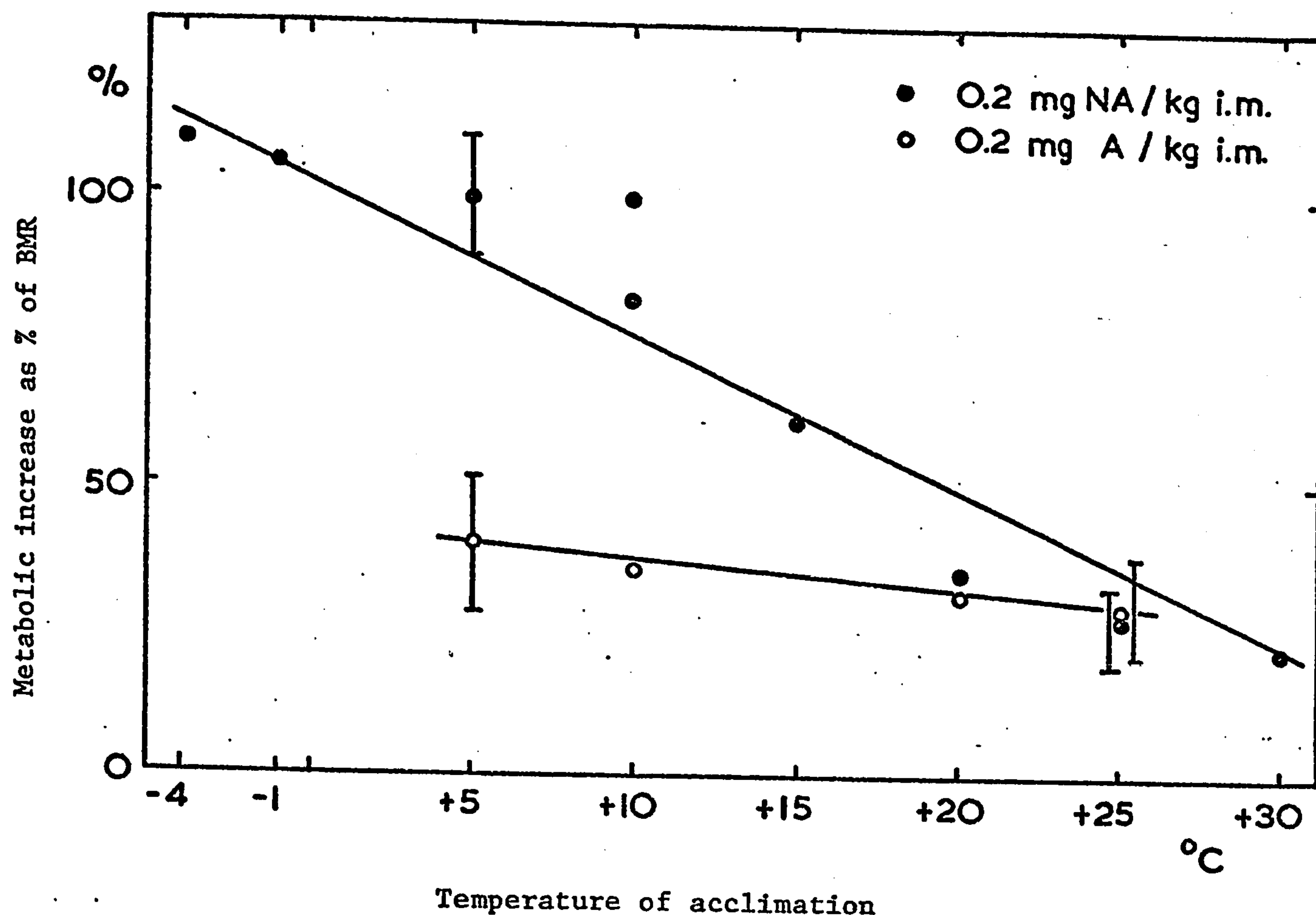


Figure 7: Effect of the acclimation temperature on the metabolic response due to intramuscular injections of noradrenaline or adrenaline in anesthetized rats, kept at different temperatures for 3 weeks prior to experiments.

Remark: Himms-Hagen has proved that there is no significant difference in the calorigenic response of the rat acclimated to 5°C either after intravenous infusion of adrenaline or noradrenaline (Himms-Hagen 1972).

(From Bartunkova et al. 1971).

of acclimation to cold reside in the sequence of hormonal and metabolic events which take place in the tissues as a consequence of the activation of the SNS, and not in the magnitude of that activation. Actually it is possible to change the time course of the development of the capacity to respond to NA by the surgical removal of interscapular brown adipose tissue (IBAT), or by inhibition of the brown adipose tissue (BAT) mitochondrial protein synthesis (Himms-Hagen 1969; 1971; 1972,b). The central role of that tissue in the development of NST will be discussed in more detail later.

e) A more direct approach was used in the experiments of Leblanc and Pouliot (1964), which showed that prolonged treatment of rats with NA increased their capacity to respond to NA as well as their cold resistance. Even if shivering in those experiments was not measured, they suggest that NA plays an important role in the development of adaptation to cold.

Thus, the measure of the calorigenic action of NA can be a very useful instrument for the measure of NST because it mimicks relatively well its two fundamental characteristics: the facultative process of its rapid switching on and off, and the slow adaptive process of the increase for the capacity for making NST during acclimation to cold.

4. RELATIVE PARTICIPATION OF VARIOUS ORGANS IN NONSHIVERING THERMOGENESIS

In order to evaluate the relative participation of individual organs in NST it is necessary to compare the increase in the total rate of oxygen consumption specifically produced by NST in a cold-exposed rat to the actual increase in the rate of oxygen consumption of individual tissues. In a recent paper Jansky states that:

> "The best and ideal technique would be one based on organ blood flows and on extraction of oxygen from the blood. However, this represents a great obstacle in small animals where cannulation of small vessels under physiological conditions is practically impossible" (Jansky 1971).

An indirect way to obtain some information is based on the logical fact that a tissue cannot consume more oxygen than is supplied to it. The problem is thus to calculate how much oxygen is delivered to individual tissues of CA rats exposed to cold. The maximum capacity of oxygen uptake by a tissue can be estimated by the rate of blood flow through that tissue multiplied by the arterial oxygen concentration, assuming that all the oxygen supplied to this tissue will be consumed by it. It is clear that this method does not provide any information about the real oxygen consumption of an individual tissue or its participation to total NST, because each tissue consumes an unknown proportion of the oxygen supplied to it, but it is very useful to eliminate certain tissues which do not receive enough oxygen, as important sites of NST.

In 1968 Jansky and Hart measured the cardiac output and the organ blood flow in WA and CA rats exposed to cold. They observed that cold-acclimation as well as cold-exposure of CA rats increased the cardiac output and that cold-exposure increased the blood flow through several tissues of WA and CA rats. Table 1 shows values of maximum oxygen consumption of rats making actively NST, i.e. the CA rats in the cold, that were calculated from the tissue blood flow data given by Jansky (Jansky and Hart 1968), knowing that the arterial O_2 concentration was 16.7ml/100ml of blood and assuming that all the oxygen supplied was consumed by the tissue. The comparison of the maximum capacity of tissue oxygen consumption with the real oxygen consumption of the CA rats in the cold which is 16ml O_2/min/rat gives the maximum capacity of a tissue to participate in NST.

TABLE I

MAXIMUM CAPACITY OF INDIVIDUAL TISSUES FOR NST IN COLD ACCLIMATED RATS EXPOSED TO COLD (9°C)

ORGAN	WEIGHT (g)	BLOOD FLOW (ml/organ/min)	Maximum capacity for O_2 (2) consumption (ml O_2/organ/min)	% of total metabolism (3) (Maximum capacity for NST)
Whole rat	317.00 (8)[1]			
Carcass	169.10 (8)	55.85 ± 15.40	9.32	58.2
Kidneys	3.84 (8)	32.40 ± 5.63	5.41	33.8
Intestine	39.30 (8)	54.03 ± 11.60	9.02	56.3
Liver	16.60 (7)	12.78 ± 2.47	2.13	13.0 (4)
Skin	49.95 (8)	13.77 ± 3.66	2.29	14.1
Total				175%
Heart	1.20 (8)	5.40 ± 1.24	0.901	5.6
Diaphragm	1.07 (8)	1.65 ± 0.62	0.275	1.7
Lung	2.32 (8)	4.90 ± 1.25	0.818	5.1
Spleen	0.87 (8)	1.72 ± 0.56	0.287	1.79
Thymus	0.58 (7)	0.66 ± 0.29	0.110	0.68
Pancreas	1.46 (4)	2.91 ± 0.49	0.485	3.03
White fat	3.02 (8)	0.76 ± 0.31	0.127	0.79
Brown fat	1.54 (8)	3.59 ± 0.60	0.599	3.74
Testes	5.60 (8)	1.68 ± 0.36	0.280	1.75
Adrenals	0.07 (5)	0.50 ± 0.30	0.088	0.55
Total				24.73%
Grand Total		191.00	31.89	199.73%

(1) Number of animals
(2) Arterial blood O_2 concentration: 0.167 ml O_2/ml blood
(3) Total metabolism: 16 ml O_2/rat/min.
(4) If the portal blood is assumed to contain .082 ml O_2/ml blood, then liver will receive a supplement of 4.43 ml of O_2/min from intestine which will raise its maximum capacity for NST to 41% and will decrease consequently the maximum capacity for intestine.

From Jansky and Hart, 1968.

From these calculations it is evident that small tissues like heart, diaphragm, lung, spleen, thymus, pancreas, white fat, brown fat, testes, adrenals cannot be important sites of NST because at best the sum of their maximum capacity for consuming oxygen represents only 24.7% of NST, and that 5 tissues : muscle, (carcass)kidneys, intestine, liver and skin could be "a priori" important sites of NST. Another important deduction is that unlike the specifically localized muscle shivering thermogenesis, NST cannot be localized in only one of those tissues because none of them has a capacity equal or superior to 100% for making it. The problem is thus to estimate the proportions of the participation of those 5 remaining tissues to NST:

A. MUSCLE

Depocas (1958; 1960,b) was the first who clarified that problem in 1958 by demonstrating in a very elegant experiment that eviscerated, curarized CA rats showed a large increase in oxygen uptake which was only slightly lower than that of the non-eviscerated CA control group (Fig. 8). However, considering that evisceration per se reduced oxygen consumption because it puts out of circulation several tissues, the increases in oxygen consumption were similar in both groups.

Depocas thus proved that the viscera, including the liver and the intestine, are not required for the switching on of NST and that extravisceral tissues are quantitatively more important sites for NST than the visceral ones. By deduction, he focussed the attention on extravisceral tissues like skeletal muscle.

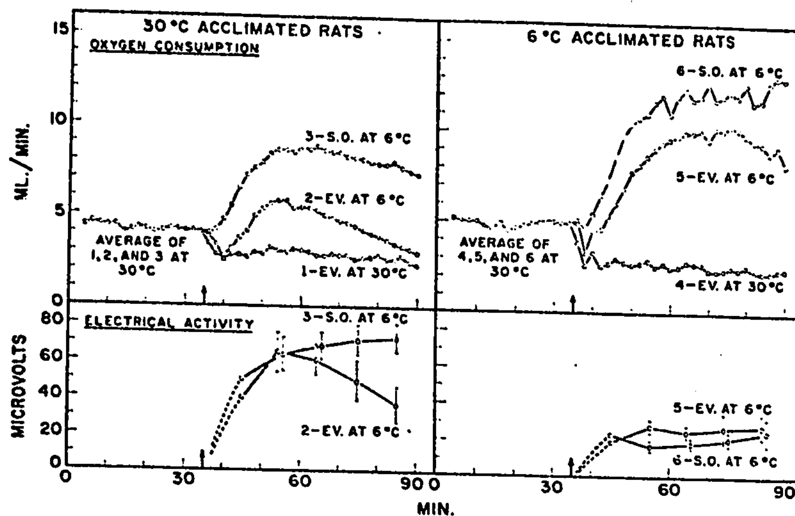


Figure 8: Persistence of nonshivering thermogenesis in functionally eviscerated rats. Average O_2 consumption and muscle electrical activity of barbital-anesthetized 30° and 6°C acclimated rats at 30°C (average O_2 consumption of all similarly acclimated rats given for interval 0 - 35 minutes), at 30° after evisceration (groups 1 and 4), at 6° after evisceration (groups 2 and 5), and at 6°C after sham-operation (groups 3 and 6) are shown. Arrow indicates time of evisceration or transfer to 6°C or both. All groups consisted of five animals except group 3 in which there were six. Evisceration consisted of tying off the rectal colon, coeliac and superior mesenteric arteries and the portal vein; this procedure effectively prevents blood flow through intestine and liver.

(From Depocas, 1958).

More direct evidence for the participation of muscle in NST was obtained in 1963 by Jansky and Hart (Jansky and Hart 1963). They measured "in situ" the arterio-venous differences in blood oxygen content and blood flow of different leg muscles of CA rats before and during exposure to cold, or during NA perfusion at room-temperature. They observed that the oxygen uptake of leg muscles increased 104% after NA infusion and 132% after exposure of the rat to cold, while the oxygen uptake of the whole rat increased 112% and 126% respectively. As the total skeletal muscle weight is about 48% of the total rat weight (Heroux and Gridgeman 1958), Jansky estimated that the contribution of muscle to NST is 50-70% (Jansky and Hart 1963; 1968). It should be noted that the authors point out that the increased oxygen consumption in leg muscles produced by NA or cold exposure was due almost entirely to the increases in arterio-venous oxygen differences and not to an increase in blood flow because in all their experiments arterial oxygenation and blood flow did not change, but venous oxygen fell. This suggests that muscle can increase its capacity for taking up oxygen out of the blood without a necessary increase in oxygen supply.

B. KIDNEYS

In contrast to muscle Jansky observed that the kidneys did not increase their oxygen uptake either after cold-exposure or NA infusion. Jansky estimated therefore that the kidneys do not participate in NST (Jansky 1971).

C. LIVER

Similar "in situ" measures of liver oxygen consumption under "in vivo" conditions seem technically difficult to realize due to the complicated vascularisation of the organ (Jansky 1971). Working with isolated rat livers from CA rats perfused with undiluted blood, Jansky showed that the liver does not increase its oxygen consumption after injection of NA to the perfusate (Jansky, 1964) but that the perfused liver can increase its oxygen consumption by increased blood flow and blood oxygenation. However, it should be noted that the perfused livers consume relatively small quantities of oxygen compared to what liver could consume "in vivo" (Jansky and Hart 1968). Moreover, Kawahata and Carlson showed in 1959, that the blood flow through the liver increased only 10% in curarized CA rats exposed to cold, and that there was no difference in the oxygen tension between the livers of WA and CA animals.

D. INTESTINE

Intestine like liver was eliminated as important site for NST by Depocas' experiment (Depocas 1958). Recently Jansky measured directly the participation of intestine to NST by blood flow measures and A-V differences between the aorta and the portal vein in CA animals, before and after exposure to cold. He did not observe any change in the A-V differences in oxygen content, but as the blood flow increased, he estimated the increase in oxygen consumption of intestine as being 64%, and the participation of intestine to NST as being less than 10% (Jansky 1971).

E. THE SKIN

Very few experiments were made with the skin. Cold exposure of

CA rats does not change the blood flow to the skin (Jansky and Hart 1968), and the separation of the skin from the leg had no significant effect on leg oxygen consumption during NA infusion of CA rats (Jansky and Hart 1963).

In conclusion, it is clear that if the muscle is the most important site of NST in the CA rat, it is not a unique site. Its participation in NST can be reasonably evaluated as 50-70% of the total NST, the rest being distributed between various tissues in which the liver, the intestine and BAT are the most important sites. Moreover, it should be noted that we are not aware of any direct demonstration that there is a development of an increased capacity for NST in the muscle or any other tissue of the rat during acclimation to cold. From the experiments of Depocas (1958) this seems a very likely possibility and a definitive answer could probably be given by comparing A-V oxygen differences in curarized or denervated muscles of WA and CA rats after cold-exposure or perfusion with NA.

5. ROLE OF BROWN ADIPOSE TISSUE IN NONSHIVERING THERMOGENESIS

Considerable evidence demonstrates that BAT is a site of heat production which is under the control of the SNS (Chaffee and Roberts 1971; Himms-Hagen 1970; Schönbaum et al. 1970; Smith and Horwitz 1969; Girardier and Seydoux 1971).

As mentioned above, the contribution of BAT to NST in the CA rats is about 5%, as calculated from blood flow data. Calculations from various similar experiments suggest that its maximal contribution cannot exceed 8 to 12% (Himms-Hagen 1972, a). In this respect it is interesting to compare the CA rat to the new-born rabbit because it seems that they represent two extremes for the quantitative contribution of BAT for NST: the surgical removal of the IBAT* in the new-born rabbit results in an immediate and important loss in the calorigenic response to cold-exposure and to NA (Hull 1965), but the same operation has no immediate effect on the CA rat, as measured by the calorigenic response to adrenaline and NA (Himms-Hagen 1969). Considering that the blood flow rates per unit weight of tissue are similar in both cases (Jansky and Hart 1968; Kuroshima et al. 1967; Jarai 1969), the explanation lies probably in the fact that the new-born rabbit has much larger quantities of BAT relative to the body weight than the CA rat (in which it represents less than one per cent of the body weight even if during the process of acclimation to cold, BAT has tripled its initial mass).

Two important qualitative functions of BAT have been proposed:

(1) Smith and Horwitz (1969) have suggested that BAT is a thermoregulator organ. This function results from its special vasculature and anatomical location. For instance, heat chanelled through the Sulzer's vein of IBAT could warm up certain spinal cord thermal receptors responsible

* IBAT = interscapular brown adipose tissue (represents about 30 % of the total brown adipose tissue).

for the suppression of shivering and for the regulation of the calorigenic response to NA during cold acclimation (Horwitz and Detrick 1971). Thus, one important function of that diffuse tissue could be the local supply of heat to certain organs. However, the regulatory function of that heat supply for the development of NST and the suppression of shivering thermogenesis was not directly established.

(2) Himms-Hagen (1970) proposed that BAT has an endocrine function which controls the development of the capacity of other organs for NST.

Fig. 9 shows that CA rats which had their IBAT tissue removed lose their adaptive state to cold more rapidly than the sham-operated group if they are maintained at room-temperature. A similar reduction of about 40% of the response to NA was observed by Leduc and Rivest (1969). This suggests that BAT could secrete a factor which influences the capacity of other tissues to respond to NA and that this factor could control the rate of deacclimation to cold (the normal rate of deacclimation to cold is shown in Fig. 6B). More direct evidence for this hypothesis was obtained when it was demonstrated that the implantation of IBAT from each rat into its peritoneal cavity considerably reduces the loss of that adaptive state (Fig. 9). The influence of the presence of the special interscapular location of brown adipose tissue on the rate of acclimation to

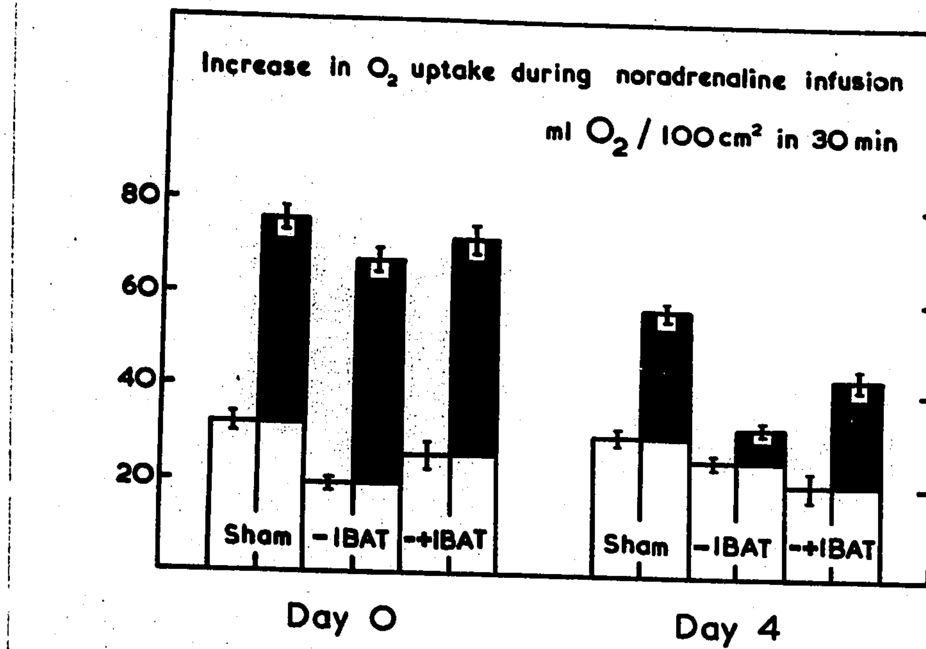


Figure 9: Effect of removal and replacement of the IBAT of WA and CA rats on the calorigenic response to NA. Values shown represent the total increase in oxygen uptake during a 30 min. infusion of NA. Each bar is the mean of 4 experiments ± standard error of the mean. CA rats are represented by the bars with the black upper portions and WA controls are represented by the open bar to the left. The blackened portion of the bar is the difference between the response of the CA rats and the response of the corresponding control WA rats; it is a measure of the acclimation to cold. CA rats had lived in the cold for 10-13 weeks; WA rats had lived at room temperature during this same period. Rats were removed from cold or warm rooms on day 0, anesthetized with ether and operated upon as follows: (i) sham-operated, (ii) IBAT removed, (iii) IBAT removed, cleaned and weighed, cut into 4 or 5 portions and replaced in the peritoneal cavity; a similar laparotomy was performed also on rats in groups (i) and (ii). Rats remained at room temperature until the infusion of NA.

(From Himms-Hagen 1970).

cold is shown in Fig. 10. It can be noted that the removal of IBAT prior to cold-exposure of WA rats delays during about one week the normal development of acclimation to cold, but does not prevent it. Thus, the interscapular location of BAT and consequently the heating through the Sulzer's vein of the spinal cord thermal receptors are not essential for acclimation to cold to occur.

It is interesting to compare these results to a recent experiment made by Flattery and Sellers (1971). These authors removed IBAT from CA rats and replaced the animals in the cold after the operation, whereas Himms-Hagen (1969) placed similar treated rats at warm temperature in order to study cold-deacclimation. They observed that during the four weeks there was no significant change in urinary excretion of the catecholamines nor in the calorigenic response to NA injection between the operated and non-operated groups of rats.

This set of experiments demonstrates that the presence of IBAT is not of critical importance in CA rats in the cold as in WA rats in the warm, but its presence is important when the WA rat is placed in the cold or when the CA rat is placed in the warm. It seems therefore that IBAT controls the mechanisms of acclimation and deacclimation to cold, but its presence is not required for the maintenance of the acclimated state.

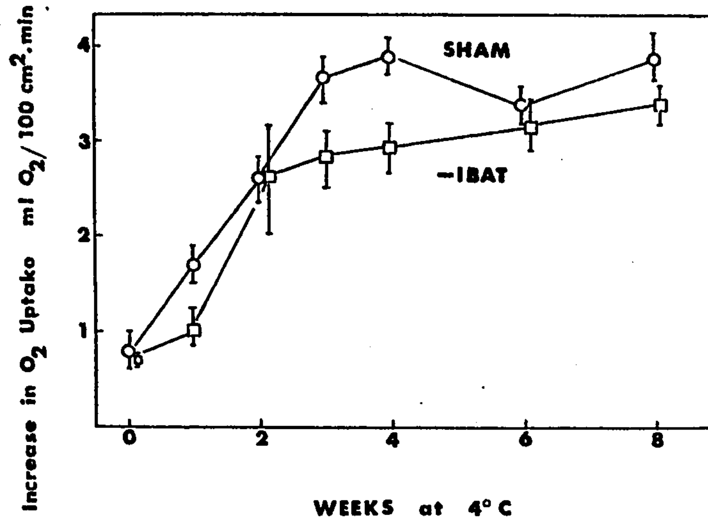


Figure 10: Effect of prior removal of IBAT on the development of the enhanced calorigenic response to NA during acclimation to cold. One week before zero time (the day on which rats were placed in the cold at 4°C) rats either had their IBAT removed under ether anaesthesia (-IBAT) or were sham-operated (SHAM). Weights of all rats at this time were: sham, 226.3 ± 20.7 g; -IBAT, 243.8 ± 26.8 g. Rats remained at 4°C for up to 8 weeks at which time they weighed: sham, 306.5 ± 24.9g; -IBAT, 334 ± 19.1 g. At 1,2,3,4,6 and 8 weeks some rats were removed from the cold; after 24 hours at room temperature they were lightly anaesthetized with sodium pentobarbital and their oxygen consumption before and during infusion with NA was measured.

(From Himms-Hagen et al. 1972, b).

6. BIOCHEMICAL CLASSIFICATION OF THERMOGENIC PROCESSES

Prusiner and Poe (1968) have proposed a biochemical classification of heat producing mechanisms based on thermodynamic, biochemical and physiological considerations. They have developed the following points:

1. The main site of oxidative metabolism in most tissues is the mitochondrial respiratory chain.
2. In most oxidations of biological foodstuffs, the change in free energy is very nearly equal to the change in enthalpy and consequently, according to the second law of thermodynamics, the change in entropy is relatively small.
3. If it is assumed that oxidation is fully coupled to phosphorylation of ADP into ATP and if the "in vivo" change in enthalpy during ATP hydrolysis is -4.7 Kcal/mole, then about 25% of the enthalpy produced when food is oxidized is conserved in ATP, the rest being released as heat.

Thus, because respiration is the bioenergetic basis of heat production and because any increase in respiration will be accompanied by a parallel increase in heat production, it is essential for the understanding of the mechanisms responsible for the increase of the metabolic rate of a rat exposed to cold, to study the nature and the origin of the factors which control the respiration of the mitochondrial respiratory chain "in vivo".

The biochemical classification of Prusiner and Poe is based on four respiratory control points:

(a) ATP degradation: The respiration is controlled at the level

of ATP degradation. The mitochondrial respiration is coupled with the phosphorylation of the phosphate acceptor and is controlled by its rate of regeneration.

(b) Oligomycin-insensitive respiration (but sensitive to uncouplers): Oligomycin inhibits partially, but not totally, respiration in coupled mitochondria and blocks ATP synthesis and degradation. The residual respiration is called oligomycin-insensitive. An uncoupler like dinitrophenol can release the oligomycin effects on respiration but not on ATP synthesis. Thus, in coupled mitochondria there exist respiratory pathways which are not coupled with phosphorylation of ADP and whose control is not understood. In this context, Lehninger showed that the active transport of divalent cations like calcium is insensitive to oligomycin but blocked by uncoupling agents (Lehninger 1970).

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

(d) Uncoupling: Uncoupled mitochondria are not affected by oligomycin or dinitrophenol but have very low P:O ratios. Their respiration seems to be mainly controlled by substrate supply.

In this classification shivering thermogenesis pertains to the category: "ATP degradation" where the shivering of muscles is the control point of respiration. Because the mechanism of NST is not understood it could in principle be classified in any of the four categories, except perhaps in a totally uncoupled mechanism, because BAT, skeletal muscle and all the other tissues actively involved in NST require also a normal supply of ATP for their development and for the maintenance of their functions. In BAT this need for ATP is even increased during acclimation to cold because the tissue increases markedly its mass and protein content during that period (Smith and Horwitz 1969).

Of the other four proposed mechanisms, the "loosening" of oxidative phosphorylation has received much support in BAT. For a review see: (Himms-Hagen 1970). Very recently it was demonstrated that in isolated BAT mitochondria a very small and specific endogenous fatty acid fraction is involved in the regulation of the coupling-uncoupling mechanism of BAT, probably through effects of allosteric nature and it was suggested that this mechanism could be involved in the NA induced respiration of the BAT (Bulychev et al. 1972; Cannon 1972).

Moreover, changes in the relative concentrations of adenosine phosphate nucleotides within the mitochondria of BAT could also regulate the coupling of respiration to phosphorylation (Pedersen and Grav 1971). In reality, both hypotheses suggest that fatty acids or nucleotides could act on the energy conserving mechanism of the mitochondrion via some conformational change in the mitochondrial membrane (Bulychev et al. 1972; Christiansen 1971).

In summary, the development of the CA state is accompanied by an increased capacity for NST and by increased calorigenic effect of NA. The skeletal muscles by their quantitative participation to NST and BAT by its regulatory function seem to be the principal tissues involved in the development of cold-acclimation. As increased capacity for NST means increased capacity for making heat, and as mitochondria are the principal sites of heat production in the cell, the initial working hypothesis of this thesis was that changes in the capacity for producing increased quantities of heat by NST could be accompanied by changes in the structure and the function of mitochondria. It was therefore decided to study the problem of mitochondriogenesis in various tissues of the rat during acclimation to cold. The mechanisms of biogenesis of mitochondria will be reviewed in the next section.

B. THE BIOGENESIS OF MITOCHONDRIA

1. INTRODUCTION

During the last decade the mechanism of the biogenesis of mitochondria has been subjected to extensive studies which have been reviewed recently by several authors (Roodyn 1968; Nass 1969; Swift 1969; Kroon 1969; Borst 1969; Borst and Kroon 1969; Getz 1970; Rabinowitz 1970; Ashwell and Work 1970).

Some of those reviews discuss only certain limited aspects of the many problems related to mitochondrial biogenesis like mitochondrial genetics, the mitochondrial biosynthesis of lipids, nucleic acids or proteins, probably because it becomes constantly more difficult to review the multitude of papers related to this ever-growing subject. However, regardless of their specialization, many of the authors have a common preoccupation which is to try to answer the old question already raised in 1890 by Altmann: "Are mitochondria self-reproducing organelles?" This was and remains a very interesting suggestion which can be justified today by two different types of evidence:

- Altmann's hypothesis was based on morphological studies which led him to suggest that mitochondria could be considered as modified bacteria living as symbiotes within the cell. The striking morphological, biochemical and functional similarities between mitochondria and bacteria have continued to accumulate

during the following years and have consolidated the theory of the bacterial evolutionary origin of mitochondria. As bacteria are self-reproducing organisms, mitochondria could share the same property. This theory is critically reviewed by Nass (1969) and Roodyn (1968).

- The phenomenon of the cytoplasmic inheritance, the discovery of mitochondrial mutants, the recognition of cytoplasmic Feulgen - positive material led to the final discovery and characterization of mitochondrial DNA. As a matter of fact, mitochondria are completely equipped to synthesize specific proteins: they have their own DNA, a DNA polymerase, ribosomes, different types of RNA (ribosomal RNA's, tRNA's, messenger RNA), a DNA-dependent RNA polymerase, amino-acyl-transfer RNA synthetases. The final demonstration of mitochondrial autonomy results from experiments which showed that mitochondria are capable of synthesizing proteins in the absence of the microsomal proteins synthesizing apparatus.

Thus, Altmann's question has to be reformulated into another series of questions: what is the degree of mitochondrial autonomy, what controls the mechanism of mitochondrial biosynthesis and replication, what are the functions of the proteins made by mitochondria, what is the relation between those proteins and the mitochondrial energy regulating mechanism, and finally what is the real advantage, if there is one, for the eukaryote cell of having compartmentalized its genetic information and protein synthesizing apparatus? Those problems will be discussed in the following sections.

2. MITOCHONDRIAL DNA AND ITS CODING CAPACITY

Mitochondria contain DNA (M-DNA) (Rabinowitz 1970; Kroon 1969; Borst and Kroon 1969) which like bacterial DNA exists in general as a circular double stranded molecule. Its molecular weight is around 1 x 10^7 daltons in the vertebrates, but is in general higher for other organisms like yeast, Neurospora crassa, or plants. There is more than one molecule of DNA per mitochondrion, 4 to 5 molecules have been proposed for liver (Borst et al. 1967), but it is not known if they are similar or not (Roodyn 1968), because physical methods like the determination of their buoyant densities and size measurement by electron microscopy are relatively insensitive indicators of genetic homogeneity. Those methods show that mitochondrial DNA is apparently homogeneous (Rabinowitz and Swift 1970), but then it is possible to wonder why the same genetic information exists in the mitochondrion in several duplicates. The problem is important for the estimation of the coding capacity of the mitochondrial DNA. If it is assumed that all molecules of DNA are similar and knowing that the size of the circular mitochondrion in mammalian mitochondria, as measured by electron microscopy, is in general 5 microns and that the base pairs are spaced 3.3 angstroms, a simple division shows that there are only 15,000 base pairs or 5,000 codons in the mammalian M-DNA, which corresponds to a molecular weight of about 10^7 daltons. Even if underestimated several times, these calculations show that mitochondrial DNA has a very poor coding ca-

pacity in comparison with E. Coli (which has 200 times more base pairs) or human haploid genome (which contains 200,000 as many). Sinclair et al. (1967) who made these calculations concluded therefore that mammalian mitochondrial DNA could code for only 5,000 AA (amino acids), or for about 30 peptides of molecular weight of 20,000. Knowing the complexity of the enzymatic and structural composition of mitochondria, it is possible to conclude, only on the basis of their limited coding capacity, that mitochondria are not totally autonomous organelles. On the contrary, the majority of their proteins should be coded by the nuclear DNA. The mitochondrial coding capacity would be totally expended if it would only code for the 30-50 different mitochondrial ribosomal proteins. It is important to note that this does not mean that mitochondria synthesize only a few proteins, because it is possible that nuclear messenger RNA could be translated on mitochondrial ribosomes just as the mitochondrial messenger RNA could be translated on the cytoplasmic ribosomes.

M-DNA can undergo a semi-conservative mechanism for its replication (Luck et al. 1964; Gross and Rabinowitz 1969,a) and it possesses an independent mechanism for DNA synthesis, since it was demonstrated that isolated mitochondria can synthesize DNA in the presence of the four nucleoside triphosphates (Brewer et al. 1967) and that dATP is actively incorporated in inner mitochondrial preparations (Kalf and Faust 1969) in a reaction which is Mg dependent, inhibited by actinomycin D or sonication and mediated by a specific mitochondrial DNA polymerase.

3. MITOCHONDRIAL DNA POLYMERASE

The mitochondrial DNA polymerase is different from the nuclear enzyme (Neubert et al. 1967) and has been partially isolated and purified from liver (Meyer and Simpson 1968; Kalf and Ch'ih 1968) and from yeast (Iwashima et al. 1969). The mitochondrial enzyme, like the nuclear one, has an absolute requirement for a DNA primer but the enzyme template complex is much more sensitive to inhibition by ethidium bromide or acriflavine than the nuclear complex, which is a very interesting property for the study of the information which is coded specifically by M-DNA (Meyer and Simpson 1969). However, it seems that the M-DNA polymerase is synthesized outside the mitochondrion, on the cytoribosomes (Ch'ih and Kalf 1969).

4. MITORIBOSOMES

Mitochondria have only a few ribosomes and this makes the isolation and the study of the constituents of these ribosomes relatively difficult to realize. Only in Neurospora crassa has it been demonstrated that the the 73 S ribosomes are unequivocally of mitochondrial origin (mitoribosomes) and that they are relatively smaller than the 78 S cytoribosomes (Kuntzel and Noll 1967; Rifkin et al. 1967).

Moreover, it is possible to observe by electron microscopy ribosomal like particles (André 1965; Swift 1965). The size and the sedimentation characteristics of mitoribosomes vary widely not only between different organisms, but even in the rat liver, where

values from 50-55 S to 83 S have been reported (Ashwell and Work 1970). Kroon (1969) has recently listed several criteria which have to be taken into consideration in order to evaluate this marked variation between the sedimentation coefficients of mitoribosomes of a same tissue; mitochondria have to be free of any microsomal contamination, the RNA:protein ratio of the mitoribosomes and the base composition of ribosomal RNA have to be determined, but probably the most important criterion is that the mitoribosomes should be able to carry out protein synthesis "in vitro" which should be sensitive to inhibitors of mitochondrial protein synthesis like chloramphenicol and insensitive to cycloheximide,(which is a strong inhibitor of the cytoribosomal protein synthesis). Thus, it is quite possible that the size of mitoribosomes differs between different species or between different tissues, but further studies are needed before any definitive conclusion can be formulated.

5. MITOCHONDRIAL RNA'S AND MITOCHONDRIAL RNA POLYMERASE

The best evidence for an intramitochondrial RNA synthesis comes from experiments which showed that mitochondria incubated in a suitable medium containing the four ribonucleoside triphosphates can incorporate them into their RNA (Saccone et al. 1969; Sumaya and Eyer 1968). Ashwell and Work (1969) and Rabinowitz and Swift (1970) have discussed the many problems of the origin of the various mitochondrial RNA's. There are several types of specific

mitochondrial RNA in different species and tissues: the 23-27 S RNA, the 16-18 S RNA, the 4 S mitochondrial transfer RNA. Molecular hybridization experiments have demonstrated that the mitochondrial ribosomal RNA is probably derived from mitochondrial DNA and the RNA of the cytoribosomes does not compete with the RNA of the mitoribosomes for sites on the mitochondrial DNA. However, it is not clear if all the different types of mitochondrial RNA are coded by mitochondrial DNA, because it was observed that mitochondrial RNA could hybridize with nuclear DNA.

By analogy with the cytosol RNA's, it can probably be assumed that the 23-27 S RNA is derived from the larger particle of mitoribosomes and that the smaller 16-18 S RNA is derived from the smaller sub-unit. The fractionation of the 4 S RNA has demonstrated that it contains several types of transfer RNA (tRNA), which are specific for AA. For instance, it was shown that Neurospora mitochondria contained at least 15 different amino acyl-t RNA synthetases and that those enzymes as well as the tRNA's have different chromatographic and physico-chemical properties from their corresponding cytosol molecules (Epler 1969). It is interesting to note that with rat liver mitochondria Buck and Nass (1969) have shown that several mitochondrial synthetases can acylate specifically the mitochondrial tRNA as well as the cytoplasmic tRNA, but the cytoplasmic synthetases were unable to acylate the corresponding mitochondrial tRNA. In Neurospora crassa, the specificity seems even greater, because it was shown that certain

mitochondrial synthetases were able to acylate only mitochondrial tRNA's and not the cytoplasmic ones (Barnett et al. 1967). It is interesting to note that in the same organism the coding properties for mitochondrial and cytoplasmic leucine tRNA are different (Epler and Barnett 1967). It is not yet known if some or all mitochondrial tRNA's are transcribed from mitochondrial DNA.

Mitochondria have an RNA polymerase but it is not fully established whether this enzyme differs or not from the corresponding nuclear enzyme, because it has not yet been solubilized and purified (Kroon et al. 1967; Luck 1964; South and Mahler 1968). The enzyme is inhibited by actinomycin D and is present in inner membrane preparations. It requires the presence of the four nucleoside triphosphates.

6. <u>PROTEIN SYNTHESIS BY ISOLATED MITOCHONDRIA AS RELATED TO THE PHYSIOLOGICAL STATE OF VARIOUS TISSUES</u>

In 1958, McClean et al. showed that isolated mitochondria are able to incorporate labelled AA into their proteins and from that time much evidence has shown that the capacity of incorporating AA is a general feature of mitochondria from various species and tissues (Ashwell and Work 1970; Roodyn 1968; Schatz 1970).

That this incorporating activity is specifically mitochondrial

and not due to microsomal or to bacterial contamination was demonstrated by several different approaches:

a) Microsomal contamination

- the activity is not inhibited by cycloheximide or by ribonuclease which completely inhibit the activity of the microsomal system, whereas it is sensitive to inhibition by chloramphenicol and several other antibiotics that do not affect microsomal incorporation.
- it is demonstrated by the use of enzyme markers that mitochondria washed four times by resuspension in the isolation medium contain about 2% of microsomal contamination (Beattie 1969).
- isolated mitochondria do not require the presence of the "pH 5 enzymes" which are essential for ribosomal incorporation (Kroon 1963).

b) Bacterial contamination

- Sandell et al. 1967 claimed that completely sterile mitochondrial preparations were unable to incorporate AA into proteins. However, several other laboratories have shown the contrary, by demonstrating that the addition of bacteria to sterile but active preparations of mitochondria does not affect appreciably the rate of incorporation, and that there was no correlation between the amount of AA incorporation and the number of bacteria present. In

fact, up to 10^5 bacteria per mg of mitochondrial protein were shown not to affect the rate of mitochondrial AA incorporation (Kroon et al. 1967).

- Beattie et al. (1967,b) questioned the composition of the medium used by Sandell et al. (1967), claiming that it lacked some factor necessary for mitochondrial protein synthesis. Moreover, they found that bacterial incorporation started to be significant after addition of 10^4 bacteria per mg of mitochondrial protein, but if isolation of mitochondria was carried out in sterile conditions, as little as 100 bacteria per ml of incubation or 30-50 bacteria per mg of mitochondrial protein could be achieved. It seems thus, that the problems of microsomal and bacterial contamination are real, but that they can be avoided if necessary precautions are taken, for example by working in conditions as sterile as possible and by washing mitochondria several times in order to avoid microsomal contamination and/or by inhibiting any possible mitoribosomal incorporation by cycloheximide or ribonuclease treatment.
- the most convincing argument that the "in vitro" incorporating activity of isolated mitochondria is not due to bacterial contamination comes from studies in which it was shown that it is related to the physiological state of the tissue from which the mitochondria were isolated as shown in Table 2.

TABLE 2

"IN VITRO" MITOCHONDRIAL AA INCORPORATION RELATED TO THE PHYSIOLOGICAL STATE OF THE TISSUE

Animal-Tissue-Reference	Physiological State & observed effect
Locusta Migratoria flight muscle Kleinow et al. 1971	Progressive decrease upon molting of the insects
Rat Liver Roodyn 1965	Mitochondria from newborn rats incorporate more AA than mitochondria from older rats
Rat Brain Klee and Sokoloff 1965	idem
Rat Liver Braun et al. 1963; Malkin, 1970	Increase after partial hepatectomy (regenerating liver)
Rat Liver Bronck 1963	Increase after administration of thyroid hormones to thyroidectomized rats
Roodyn et al. 1965	idem
Volfin et al. 1969	idem
Thoracic Muscles of the Tobacco Horn Worm Chan and Richardson 1969	Increase during development of Pupae into adult Moths
Rat Heart Shahab L. and Wollenberger A. 1970	Increase in hypertrophying hearts with aortic constriction
Rabbit Liver, Brain & Heart Hamberger et al. 1969	Increase in brain and heart after acute swimming exercise but no change in liver
Neurospora crassa Greenawalt (personal communication)	Increase during Morphogenetic development
Rat Intrascapular Brown Adipose Tissue Present study	Increase during acclimation to cold

It seems thus that these "in vitro" experiments reflect at least partially the "in vivo" situation, principally in studies where there is increased development of a tissue like in thoracic muscle, Neurospora crassa cells, BAT during acclimation to cold, regenerating rat liver and hypertrophying hearts. This was directly confirmed in this study as well as in Greenawalt's experiments with Neurospora crassa (1972), where "in vivo" and "in vitro" measurements of mitochondrial protein synthesis showed similar, but not identical increases after development of the cells. However, the "in vitro" AA incorporation activity is merely a reflection of the "in vivo" situation as demonstrated by Hawley and Greenawalt (1970), who observed in parallel experiments that the "in vivo" rate of AA incorporation of Neurospora crassa cells is much higher than the "in vitro" rate.

It is interesting to note that isolated mitochondria are able to incorporate not only AA, but also precursors of nucleic acids (reviewed by Rabinowitz 1970), of phospholipids (reviewed by Getz 1970) and of glycoproteins or glycolipids (Bosmann and Martin 1969; Bosmann and Case 1969; Bosmann 1971). This suggests that mitochondria possess an individual specific system responsible for the synthesis of different types of macromolecules which are involved in their structure and function.

7. THE LOCALIZATION OF MITOCHONDRIAL ENZYMES

Ashwell and Work (1970, Borst (1969, b) and Ernster and Kuylenstierna (1970,a) have reviewed the localization of more than 40

different enzymes between the four main compartments of mitochondria: the outer membrane, the inner membrane (and cristae), the matrix and the space between the inner and outer membranes. The localization of several of those enzymes like monoamine oxidase or the enzymes of the citric cycle is actually in dispute between Green's group and several other laboratories (for a comprehensive review of the problem, see Ernster and Kuylenstierna 1970, b). The problem seems to arise from the fact that at least three different methods (or combinations of those methods) were used by different laboratories to fractionate the mitochondria: "mechanical" methods based on the disruption of mitochondria by swelling-shrinking procedures followed in general by sonication, various "chemical" methods based on the use of digitonin or other compounds, or the phospholipase "enzymatic" method. It is clear that all those methods use rather drastic and quite different fractionation procedures, whereas the forces that hold many of those enzymes together are relatively weak. However, there is general agreement that the inner membrane and cristae fractions contain all the enzymes of the electron transport chain (succinate dehydrogenase, cytochrome oxidase, the ATPase complex, various coupling factors and several cytochromes) and that the majority of the more soluble enzymes of the tricarboxylic acid cycle are in the matrix.

The general picture that emerges from these localization studies is that the four main compartments of mitochondria have totally

different enzymatic compositions and that the inner membrane and the matrix constitute the mitochondrion in the functional sense, because they contain all the enzymes involved in substrate oxidation, respiration and energy conservation. Several authors have discussed the many biochemical, structural and functional similarities between the outer mitochondrial membrane and the endoplasmic reticulum on one hand (Schnaitman 1969; Sottocasa et al. 1967), and the inner mitochondrial membrane and the bacterial membrane on the other hand (Nass 1969), which suggest "a priori" that the outer mitochondrial membrane could at least partially originate from the endoplasmic reticulum, whereas the synthesis of the inner mitochondrial membrane could be under a relatively autonomous mitochondrial control. Several other experimental facts which will be discussed later support that idea.

Very recently, Greenawalt and Decker (personal communication) have shown that mitoplasts, which are mitochondria lacking the outer compartment, but containing within the inner membrane all the matrix proteins, carry out oxidative phosphorylation, exhibit acceptor control and energy-dependent conformational changes, and have thus demonstrated that the presence of the outer membrane is not directly required for the functioning of oxidative phosphorylation. The outer membrane, which is readily permeable to substances of low molecular weight, has a rather diversified enzymatic composition: "Rotenone-insensitive" NADH - cytochrome c reductase, NADH - cytochrome b_5 reductase, Monoamine oxidase,

ATP-dependent fatty acyl-CoA synthetase, Glycerophosphate-acyl transferase, phospholipase A_2, various transferases, etc. This suggests several different functions for the mitochondrial outer compartment which remain to be determined (Ernster and Kuylenstierna 1970,a). As the localization of many enzymes in various mitochondrial compartments is known, and as it was demonstrated that mitochondria have an individual and specific apparatus for making proteins, the next question which arises is to know which of these enzymes are made by mitochondria.

8. THE SEARCH FOR THE PROTEINS MADE BY THE MITOCHONDRION

It was already discussed before that mitochondria have a very limited genetic information capacity due to the small size of their DNA, so that only a few proteins or peptides could be expected to be coded by the mitochondrion. In higher organisms, at least three different experimental approaches have been used:

a) Amino Acid incorporation in isolated mitochondria

Proteins or polypeptides synthesized by isolated mitochondria are likely to be made on the mitochondrial ribosomes if the mitochondria are relatively free of microsomes which have in general a high capacity of incorporating AA "in vitro". This can be achieved by washing mitochondria several times with the isolation medium and/or incubating them with specific inhibitors of microsomal protein synthesis. However, this technique is not

telling us anything about the mitochondrial origin of the necessary messenger RNA which codes for those proteins.
No conclusion can be made about the proteins which are not synthesized by the mitochondrion "in vitro" because it is possible and even likely that during the isolation procedures several fundamental steps of the mitochondrial AA incorporation system have been affected.

b) <u>The "in vivo" rate of amino acid incorporation</u>

The rationale underlying this technique is that proteins synthesized by mitochondria should become labelled before the proteins synthesized by the extramitochondrial system which have to be transferred from the cytoplasm to the mitochondrion before being incorporated in its structure. If this technique has all the advantages of the "in vivo" procedures, it has also its defects:

- the mitochondrial, the microsomal and several other AA pools of precursors are unknown and by consequence their eventual influence on the rate of AA incorporation cannot be estimated.
- permeability problems could also affect the results as the injected AA has to pass through several membranes before being incorporated into the mitoribosomes. This is one of the reasons why intravenous injections have to be preferred to intraperitoneal or any other kind of injection.
- the origin of some mitochondrial proteins which are synthesized very slowly (more slowly than the microsomal proteins) cannot be detected by this kind of technique either. That certain important mitochondrial proteins are synthesized very slowly is suggested by the fact that the half-lives of the majority

of mitochondrial components (DNA, various proteins and phospholipids) is expressed in days (or weeks for tissues like muscle or brain) and also that in liver (but not in muscle) microsomal incorporation seems more active than the mitochondrial one.

c) Methods which use specific inhibitors of protein synthesis

The use of different inhibitors for the study of protein biosynthesis in bacteria, chloroplasts and mitochondria has been reviewed by Roodyn and Wilkie (1968), Borst et al.(1969,a) and by Kroon(1969). The most useful inhibitors have been choramphenicol and oxytetracycline as opposed to cycloheximide, because they are specific in inhibiting mitochondrial protein synthesis. Chloramphenicol, erythromycin and oxytetracycline have been reported to inhibit mitochondrial protein synthesis in a wide range of mitochondrial fractions prepared from animal cells, yeast and Neurospora crassa. There is a very interesting connection between bacterial ribosomes which are of the 70 S type and the mitoribosomes which are both sensitive to this type of drug and insensitive to cycloheximide. In 1966, Clark-Walker and Linnane suggested that these antibiotics may block protein synthesis at the level of the mitoribosomes in a similar way as they do it at the level of the bacterial ribosome. These authors have demonstrated that chloramphenicol inhibits the synthesis of cytochromes a and b in yeast cells and alters the structure of mitochondria: it was possible to see by electron microscopy only "mitochondrial profiles" i.e. mito-

chondria with an outer but not an inner membrane. Moreover, the effects on mitochondrial synthesis were reversible when the cells were removed from the presence of chloramphenicol (Clark-Walker and Linnane 1967). These studies were extended to the antibiotic cycloheximide which is a potent inhibitor of protein synthesis in eucaryotic cells, but has no inhibitory effects on the bacterial ribosomes. Using concentration of cycloheximide which inhibited yeast microsomal protein synthesis, the authors showed, that the formation of cytochromes a and a_3 was not preferentially affected and demonstrated that yeast mitochondria are made by two different protein synthesizing systems.

Chloramphenicol and cycloheximide are now widely used to study the origin of various mitochondrial proteins, but there are two problems in analyzing the effects of these drugs: 1) it is possible that the two protein synthesizing systems which cooperate in the formation of the mitochondrion do not operate independently of each other (logically they should not) and by inhibiting one, the other is consequently affected; 2) there exists always the possibility that these drugs especially when used at high concentrations may have several side effects.

As none of these methods is free of criticism, the combined use of several of them represents the most fruitful approach for the search of the proteins made by the mitochondrion as shown in the following discussion: Some twenty years ago Hultin (1950) and Keller (1954) observed that mitochondria were able to incorporate AA into their proteins "in vivo". In 1963, Truman reported

that particles associated with mitochondrial membranes obtained by three different fraction procedures were the site of the most rapid incorporation of AA "in vivo". In 1966, Beattie et al. fractionated liver and kidney mitochondria on the basis of the differential solubilities of their proteins into groups of protein designated water-soluble proteins, cytochrome c (proteins soluble in KCl 0.12 M), contractile proteins (proteins soluble in 0.6 M KCl); the remaining proteins were dissolved in detergents and fractionated with ammonium sulfate into groups of proteins designated as"structural"proteins and"other cytochromes". It was demonstrated that 2-5 min. after the injection of radioactive leucine, the specific activities of the readily soluble group of proteins were lower than the specific activities of the unfractionated mitochondria, while at longer times, the specific activities of different fractions were approximately identical. These results suggested that those protein components which are most easily removed from the mitochondrial structure and which have initial lower specific activities than those components which require extensive treatment with detergent for their extraction are synthesized outside the mitochondria and subsequently incorporated into the mitochondrial structure. Thus, the membrane bound mitochondrial insoluble proteins appeared to be synthesized on the mitoribosomes. This interpretation was strengthened when it was shown that isolated mitochondria incorporate labelled AA into an insoluble membrane fraction whereas negligible incorporation occured into the readily soluble mitochondrial proteins. (Beattie 1967,a).

The next step in the search for the protein made by mitochondria was the demonstration that the majority of the label was incorporated "in vitro" in an insoluble inner membrane fraction corresponding to Criddle's "structural" protein (Criddle 1962; Kadenbach 1966, Beattie 1967,a). However, it was demonstrated that the "structural" protein fraction can be resolved by gel electrophoresis into several components and recent evidence has shown that this fraction contains principally Racker's F1 coupling factor (the mitochondrial ATPase) in a denatured insoluble state (Racker 1970; MacLennan 1970; Schatz 1970) (The mitochondrial ATPase can readily be solubilized by a different technique than that used by Criddle). In addition, Kalf and Grece (1964) have suggested that mitochondrial "contractile protein" was also a product of mitochondrial protein synthesis (Ohnishi and Ohnishi 1962; Neifakh and Kazakova 1963). The notion of a mitochondrial "contractile" protein with the same properties as described by Ohnishi and Ohnishi (1962), like the notion of a mitochondrial "structural" protein, has been disputed by several groups (Vignais et al. 1963; Conover and Barany 1966; MacLennan 1970), but the fact remains that isolated mitochondria incorporate AA mainly into these "structural" and "contractile" fractions which have been shown, by gel electrophoresis, to contain dozens of different proteins. In an attempt to identify those proteins made by the mitochondrion, Beattie (1971) showed that isolated liver mitochondria do not incorporate AA into a protein associated with any of several purified fractions containing the oligomycin-sensi-

tive ATPase, the cytochromes $b-c_1$, the cytochrome oxidase complex and the majority of the proteins associated with the "structural" protein fraction. Most of the radioactivity was incorporated in a "membranous, heterogeneous, insoluble" fraction which contained less than 10% of the total mitochondrial protein and which did not migrate on gel electrophoresis. Those results suggested that the proteins made by the mitochondrion were among the largest and the least soluble ones. However, it is very possible, as Beattie admits it, that the "structural" protein fraction contained mainly **denatured** proteins which could not be well dissolved by the solubilization procedures used for gel electrophoresis.

Other experiments, based on the use of different antibiotics, showed that chloramphenicol inhibits "in vivo" the formation of membrane-bound cytochromes $a-a_3$ and c_1 in yeast (Clark-Walker and Linnane 1967) and in mammalian cells (Pious 1970; Firkin and Linnane 1969). These observations led to the suggestion that cytochrome oxidase was made by the mitochondrion which is in apparent contradiction with Beattie's results. The solution came when it was realized that cytochrome oxidase is an enzymatic complex and not a single protein. Chen and Charamlampous(1969) followed both haem protein synthesis and enzymatic activity during yeast derepression. As it was shown by immunological methods that anaerobic yeast cells("petite"mutation) contain cytochrome oxidase apoenzymes (Tuppy and Birkmayer 1969), Chen and Charam-

lampous(1969) suggested that apoenzymes of cytochrome oxidase could be converted into an active enzyme during yeast glucose derepression. They found that the cycloheximide-sensitive system (the cytoribosomes) and the chloramphenicol sensitive system (the mitoribosomes) had to cooperate to produce a functional enzyme. As the appearance of a functional enzyme was also inhibited by acriflavine, the authors concluded that the apoproteins synthesized on the cytoribosomes had to form a complex with some membrane protein synthesized on the mitoribosomes and coded by M-DNA to become fully active. In this context, Beattie (1971) showed that although no radioactivity was associated with the two major bands of purified cytochrome oxidase after gel electrophoresis, significant counts were observed in a minor slow moving band. Beattie suggested that this small protein could act as a regulatory control in the formation of a functional cytochrome oxidase synthesized on the cytoribosomes. Recently, Tzagaloff (1971,a and 1971, b) and Schatz (1972) have studied respectively the biosynthesis of the ATPase complex during yeast derepression from glucose and the biosynthesis of cytochrome oxidase during yeast adaptation to oxygen. These authors have shown that the majority of the proteins participating in the structure of the ATPase complex and the cytochrome oxidase complex were synthesized on the cytoribosomes, but the activity of these enzymes was strictly dependent on some proteins made by the mitochondrion. These experiments will be discussed in more detail later, but the general picture that emerges presently, is that the mitochondrion synthesize several unidentified, relatively insoluble proteins which are localized mainly in its inner membrane and which participa-

te in the structure and functions of at least two enzymes directly involved in the mitochondrial respiratory chain and oxidative phosphorylation.

9. QUANTITATIVE ESTIMATIONS OF MITOCHONDRIAL PROTEIN SYNTHESIS "IN VIVO"

Direct measurements of the quantity of proteins participating in the mitochondrial structure and made on the mitoribosomes have been recently realized with Neurospora crassa cells (Hawley and Greenawalt 1970) and yeast (Kellerman et al. 1971). Both measurements are based on the "in vivo" estimation of the cycloheximide-resistant protein synthesis and they both agree that the mitochondrial protein synthetic capacity is very poor (14.5% for Neurospora crassa cells and 4-13% in yeast depending on the degree of catabolite repression of the cells). Thus, direct measurements confirmed what was predicted from theoretical calculations based on the estimation of the mitochondrial DNA genetic capacity, that mitochondria make only a few of their own proteins.

10. THE RELATION BETWEEN MITOCHONDRIAL PROTEIN SYNTHESIS AND MITOCHONDRIAL FUNCTION

The existence of a direct link between mitochondrial protein synthesis and the activity of respiratory enzymes was recently suggested by at least three different laboratories working with three different systems: the study of yeast respiratory adaptation (or the transformation of promitochondria into mitochondria), the

formation of mitochondrial ATPase during yeast derepression from glucose and the biogenesis of Neurospora crassa mitochondria during germination of the spores.

a) Neurospora crassa development

Greenawalt et al. (1972) have studied changes in mitochondrial function occurring prior to and during germination of Neurospora crassa. The conidia are known to have low or absent mitochondrial enzyme activities, and energy metabolism, which is the main mitochondrial function, could therefore be fundamental to the initiation of the germination and growth of this obligatory aerobe. Greenawalt et al. (1972) showed that the dramatic increase of the ability of Neurospora mitochondria to respire and to catalyze coupled phosphorylation after germination could be correlated with an increased mitochondrial protein and phospholipid synthesis. They suggested that the "in vivo" mitochondrial protein synthesis which is cycloheximide insensitive but inhibited by chloramphenicol could contribute to the increase in respiration and phosphorylation capacity of the mitochondrial membranes. However, their attempts to examine the proteins made on the mitochondrial ribosomes, by gel electrophoresis, were complicated by the active proteolysis of their mitochondrial preparations as demonstrated by the breakdown of a standard enzyme into smaller polypeptide units when mixed with their cell preparations.

b) Yeast derepression

Tzagaloff studied the biogenesis of the ATPase complex during yeast derepression (Tzagaloff 1971, a; 1971, b). The mitochon-

drial ATPase was specifically identified by Racker as a component of the headpiece of the particles seen by electron microscopy attached to the inner face of the mitochondrial inner membrane (Racker 1970). The ATPase complex is made at least of an oligomycin insensitive ATPase (F_1), an oligomycin sensivity conferring protein (OSCP), a juncture protein (which is the least soluble protein of the complex, but which is essential to its activity) and an ATPase inhibitor. As in the case of cytochrome oxidase, there is also a necessity for the presence of various phospholipids for optimal enzymatic activity. Tzagaloff showed first that there is an increase in ATPase and electron transfer activities during yeast derepression and that this increase in mitochondrial ATPase is attributable to the synthesis of a new enzyme rather than activation of a preexisting one. In further experiments he demonstrated that chloramphenicol added during derepression induced the accumulation of a soluble ATPase (F_1) in the cell sap and that cycloheximide inhibited the synthesis and the consequent accumulation of that enzyme. Tzagaloff concluded that the mitochondrial F_1 and the OSCP are synthesized on the cytoribosomes. However, since F_1 and OSCP synthesized in the presence of chloramphenicol are not assembled into a functional ATPase complex, he suggested that the insoluble juncture protein could be a product of mitochondrial synthesis. Thus, mitochondrial ATPase bound to the mitochondrial inner membrane is sensitive to oligomycin whereas purified ATPase (F_1) and the

cytoplasmic ATPase (which is presumably identical to F_1) obtained after chloramphenicol treatment are both insensitive to oligomycin, and if the cytoplasmic ATPase (F_1) is combined with F_1 deplated mitochondrial membranes it acquires oligomycin sensitivity. Tzagaloff designed a somewhat complicated system for the assay of the juncture protein which consisted essentially in the measure of the reconstitution of an oligomycin sensitive ATPase in the presence of an excess of F_1, OSCP, and submitochondrial particles containing the juncture protein. Tzagaloff also showed that although chloramphenicol and cycloheximide both inhibited juncture protein activity, the levels of juncture protein were increased dramatically if the cells were incubated first in the presence of chloramphenicol (which produced a cytoplasmic accumulation of F_1 and OSCP) and then in the presence of cycloheximide. If the addition of the inhibitor was reversed, such effects were not observed. The fact that in the presence of cycloheximide there was increased levels of juncture protein was interpreted by Tzagaloff as indicating that juncture protein is synthesized on the mitoribosomes but that products of the cytoplasmic system such as F_1 and OSCP exert a control on mitochondrial protein synthesis. It is clear that this hypothesis deserves further evaluation, but it has the advantage of pointing out some of the most fundamental problems of mitochondrial biogenesis: if products of the cytoplasmic protein synthesizing system control mitochondrial protein synthesis, logically and by analogy, the mitochondrial protein synthesizing system should also control the cytoplasmic system. This second type of control was recently demonstrated by Schatz (1972) and will be discussed in the following section.

c) Yeast respiratory adaptation

Yeast cells grown anaerobically lack cytochromes $a-a_3$, b, c_1 and c, are respiratory deficient but possess typical mDNA, "Structural" protein, and even an oligomycin-sensitive ATPase. They have mitochondrial-like structures which are called promitochondria. Schatz (1970) proved by the use of double labeling techniques that the promitochondria are precursors of the functional mitochondria during yeast respiratory adaptation and that the adaptation requires a close cooperation of the two genetic systems of the cell. Moreover, he showed that the promitochondrial protein synthesizing system responds to oxygen and is repressed by glucose even if cytoplasmic protein synthesis is inhibited by cycloheximide. Since the synthesis of the oxygen-controlled proteins is inhibited by chloramphenicol or erythromycin in the presence of cycloheximide, these proteins are translated on mitochondrial ribosomes and they are probably coded by mitochondrial DNA as Schatz observed that acriflavin inhibits all mitochondrial or promitochondrial translation products (acriflavin inhibits specifically the "in vivo" transcription of yeast M-DNA). He demonstrated also that these proteins are not aberrant proteins and that they are obligatory intermediates of respiratory adaptation in "delayed adaptation experiments"; either chloramphenicol alone or cycloheximide alone prevent respiratory adaptation but if the cells containing promitochondria are first aerated in the presence of cycloheximide and then transferred after washing in the presence of chloramphenicol, a significant adaptation occurs whereas the reversal of the antibiotic addition does not allow adaptation.

It was also proved that this delayed adaptation requires the presence of oxygen during both incubations and is prevented if the first incubation is carried out in the presence of both inhibitors and not cycloheximide alone. Thus, it seems that during the first incubation, some oxygen-induced intermediates of respiratory adaptation synthesized by the mitoribosomes can accumulate but cannot by themselves cause the emergence of respiration unless complemented by oxygen induced products made on the cytoribosomes. Those results indicate that there should be a close cooperation between the two protein synthesizing systems involved in mitochondrial formation, that the two systems can be partially separated in time but that either the sequence of events requires first a stimulation by oxygen of a specific class of proteins made on the mitoribosomes, or that a product of promitochondrial protein synthesis controls the formation or the accumulation of at least one product of the cytoplasmic system essential for adaptation. However, one of the most interesting points of Schatz's discussion (1972) is:

> "At first sight it may be surprising that an oxygen control of protein synthesis could also be detected in fully developed yeast mitochondria; these organelles possess oxygen inducible components and should no longer respond to oxygen. It should be recalled, however, that we measured cycloheximide resistant labeling always with nongrowing cells whose turnover of endogenous proteins was probably increased by the "stepdown" conditions during labeling. We may, therefore, assume that most of the incorporation of labeled leucine into protein occurred via turnover of pre-existing proteins. Under such conditions, an effect of oxygen on protein synthesis should be detected even in the absence of net synthesis."

As oxygen can apparently induce similar proteins in promitochondria as well as in fully developed mitochondria, and as this effect was measured in exactly identical conditions, it can be postulated that at least some of these proteins are not related to the development of promitochondria into mitochondria but are a consequence of the presence of oxygen and are directly or indirectly related to a higher activity of the electron transport chain. As it is possible that oxygen induces the development of promitochondria into mitochondria by controlling mitochondrial protein synthesis, it is also possible that, once the mitochondria are fully developed, oxygen continues exerting its regulatory function at the level of mitochondrial protein synthesizing system. In the absence of oxygen, yeast mitochondria "degenerate" into promitochondria and in the presence of oxygen they do not. In reality, oxygen controls the capacity of yeast mitochondria to consume it. Its presence develops and maintains that capacity and its absence inhibits it.

11. THE MECHANISMS OF MITOCHONDRIAL BIOGENESIS

Various mechanisms have been proposed for mitochondrial biogenesis. Schatz classifies them in three main categories (Schatz 1970):

a) Mitochondrial formation from other organelles or intracellular structures;

b) "De novo" mitochondrial formation;

c) Mitochondrial formation by growth and division.

a) Mitochondrial formation from other organelles, which has received some support in the past, mainly on the ground of morphological studies, seems presently unlikely in regard of the great differences in structure, chemical and enzymatic composition, which exist between the mitochondria and other intracellular structures or organelles. Moreover, the existence of a specific mitochondrial genetic and protein synthesizing systems indicates that mitochondria possess a certain autonomy. However, as was discussed before, the cytoribosomes of the endoplasmic reticulum participate in the synthesis of the majority of the mitochondrial proteins and probably also in the majority of mitochondrial phospholipids (Getz 1970). It is also possible that despite many differences the outer mitochondrial membrane could originate, at least partially, from the endoplasmic reticulum (Nass 1969).

b) The strongest argument against a "de novo" mitochondrial formation derives from Schatz' experiments which were discussed before and in which it was demonstrated that yeast promitochondria are the direct precursors of fully developed mitochondria, upon exposure of anaerobically grown cells to oxygen. Similarly, the anaerobic growth of yeast is associated with a de-differentiation rather than a complete loss of the mitochondria (Schatz 1970).

c) The unique cinematographic studies made by Frederic with fibroblasts (Frederic 1958) suggest that mitochondria are extremely motile structures which continously change shape and some-

times coalesce with one another or with other organelles like the nucleus. Therefore, the numerous electron-micrographs showing "dividing" mitochondria can be equally interpreted as showing mitochondria in the process of fusing with another mitochondria. However, the relative independence of mitochondria and nuclear DNA (as shown by their turnover differences (Gross et al. 1969) strongly indicates that mitochondria can be formed by division or replication of a mitochondrial precursor. The experiments of Luck (1963; 1965,a;1965,b) made with Neurospora mutants requiring choline for growth can be considered as a conclusive proof that Neurospora crassa mitochondria are formed by growth and division. The mitochondrial lipids of the mutants were labelled with ^{3}H-choline and then transferred to a medium containing cold choline. Electron-microscope radioautography showed that the mitochondria were randomly labelled suggesting that they were produced by division of preexisting mitochondria (Luck 1963). In further experiments (Luck 1965,b) it was shown that mutants grown in a medium relatively deficient in choline contain less lipid and have therefore a higher density than normal mitochondria, but their lipid contents could increase after transfer to a choline-rich medium. The analysis of the mitochondrial populations by sucrose density-gradient centrifugation before, during and after they had regained their normal phospholipid content demonstrated the presence of only one mitochondrial population and not of two populations of lipid-poor and lipid-rich mitochondria as compatible with a "de novo" synthesis (Luck 1965,b).

Initally it was believed that mitochondria turned over as a unit. (*) This hypothesis was essentially based on the observations of Fletcher and Sanadi (1961) who observed that three different rat liver mitochondrial fractions and the mixed lipids turned over at the same rate. The results of several studies which followed the work of Fletcher and Sanadi are summarized in Table 3. This table gives quoted values for the half-lives of mitochondrial DNA, phospholipids, mitochondrial protein fractions and enzymes in various tissues of the rat. We have also included in this table some results made with warm-acclimated (WA) and cold-acclimated (CA) rats taken from the literature and from this present study.

(*) See Appendix

TABLE 3

ORGAN AND CONSTITUENT	HALF-LIFE (in days)	REFERENCE
Liver		
Lipids	10.6	Fletcher and Sanadi, 1961
Insoluble protein	10.8	
Cytochrome c	9.7	
Total mitochondrial proteins	8.4	Beattie et al., 1967, c
Water-soluble proteins	8.6	
Cytochrome c	8.6	
Contractile proteins	8.4	
Structural proteins	8.8	
Other cytochromes	8.2	
H_2O-soluble protein	4.2-5.1	Swick et al., 1968
0.9% KCl-soluble protein	4.5-5.0	
0.6 M KCl-soluble protein	4.6-5.3	
13% $(NH_4)_2SO_4$ fraction	4.3-5.3	
35% $(NH_4)_2SO_4$ fraction	3.9-4.8	
Residue	4.7-8.8	
Alanine Amino transferase	1.7-1.0	
Insoluble protein	9.0	Bailey et al., 1967
Phospholipids	10.0	
Mitochondrial DNA	7.1	Neubert et al., 1967
Mitochondrial DNA	No turnover	Nass, 1967
Mitochondrial DNA	9.1	Gross et al., 1969, b
Cardiolipin	11.5	
Lecithin	10.0	
Phosphatidyl ethanolamine	9.9	
Sphingomyelin	11.4	
Outer membrane	7.0	Beattie et al., 1969
Inner membrane	8.4	
Insoluble protein	10.2	
Outer membrane	4.2	Brunner et al., 1968
Inner membrane	12.6	
Soluble protein	11.6	
Outer membrane Inner membrane	Outer half-life shorter than inner	de Bernard et al., 1969

cont...

Table 3 cont.

ORGAN AND CONSTITUENT	HALF-LIFE		REFERENCE
Total proteins		6.8-9.0	Arias et al., 1969
Cytochrome c		10.5	Kadenbach, 1969
Total mitochondrial proteins		9.3	Menzies and Gold, 1971
	WA	CA	
Whole mitochondrial proteins	7.0	7.8	Present study
Water-soluble proteins	8.1	7.5	
Cytochrome c	6.1	6.1	
Contractile proteins	5.9	6.6	
Structural proteins	6.9	6.6	
Other cytochromes	6.2	5.1	
Whole mitochondrial proteins	6.0	7.6	Lusena and Depocas, 1967
Kidney			
Total mitochondrial proteins		8.7	Beattie et al., 1967,c
Water-soluble proteins		6.0	
KCl-soluble proteins		7.6	
Structural proteins		9.0	
Other cytochromes		8.4	
Mitochondrial DNA		10.1	Gross et al., 1969
Cardiolipin		11.1	
Lecithin		10.0	
Phosphatidyl ethanolamine		10.0	
Cytochrome c		11.5	Kadenbach, 1969
Total mitochondrial proteins		10.9	Menzies and Gold, 1971
	WA	CA	
Total mitochondrial protein	7.3	6.7	Present study
Water-soluble protein	5.9	5.3	
Cytochrome c	7.0	6.8	
Contractile proteins	10.7	8.9	
Structural proteins	9.7	8.8	
Other cytochromes	10.1	7.2	

Cont.

Table 3 cont.

ORGAN AND CONSTITUENT	HALF-LIFE		REFERENCE
Skeletal muscle			
Cytochrome c		43.0	Kadenbach, 1969
	WA	CA	
Cytochrome c	13.8	14.8	Depocas, 1966
Total mitochondrial protein	22.7	11.7	Present study
Water-soluble proteins	15.3	11.06	
Contractile proteins	21.6	8.1	
Structural proteins	30.9	13.4	
Other cytochromes	21.7	16.9	
Brown Adipose Tissue			
Total mitochondrial proteins	12.5	7.5	Present study
Water-soluble proteins	12.8	8.7	
Structural proteins	12.2	7.4	
Other cytochromes	8.0	12.0	
Brain			
Phosphatidyl ethanolamine	22,34,35		Cuzner, 1966
Lecithin	11,19,23		
Cardiolipin + Phosphatidic acid	73.7		
Phosphatidyl serine	27		
Phosphatidyl inositol	29,34		
Whole mitochondria	26.3		Beattie, 1967, c
Water-soluble proteins	17.9		
Water-insoluble proteins	31.4		
Mitochondrial DNA	31.0		Gross et al., 1969, a
Cardiolipin	42.4		
Lecithin	48.7		
Total mitochondrial proteins	24.4		Menzies and Gold, 1971

Cont...

Table 3 cont.

ORGAN AND CONSTITUENT	HALF-LIFE	REFERENCE
Heart		
Mitochondrial DNA	6.7	Gross et al., 1969, a
Lecithin	10.5	
Phosphatidyl ethanolamine	17.4	
Total mitochondrial proteins	17.8	Menzies and Gold, 1971
Cytochrome c	43.0	Kadenbach, 1969
Intestinal Mucosa		
Total mitochondrial proteins	17.6	Menzies and Gold, 1971
Lung		
Total mitochondrial proteins	16.6	Menzies and Gold, 1971
Testes		
Total mitochondrial proteins	12.6	Menzies and Gold, 1971

The first deduction which can be made from the analysis of Table 3 is that the values for the half-lives of mitochondrial DNA, total proteins, different protein fractions, phospholipids, enzymes are not only different when compared between various tissues of the rat, but there is also much variation between the half-lives of several components of mitochondria in specific tissues. It seems, therefore, that the hypothesis of Fletcher and Sanadi (1960) is not valid for the mitochondrion considered as an entity, but it may still have some validity if it is restricted to a unit of the inner mitochondrial membrane like the mitochondrial "structural" protein or some essential lipoprotein complex with structural and possibly also with catalytic properties (as the notions of "structure" and "catalysis" do not necessarily have to be opposed).

The second deduction is that there should be very important quantitative and possibly qualitative differences in the mechanisms of the biogenesis and turnover of muscle or brain mitochondria that turn over much more slowly than liver, kidney or BAT mitochondria. It is possible that there exist important differences between mitochondria of different tissues of the rat, which are related to their function in those tissues. The main function of muscle mitochondria is to supply enough ATP for muscle contraction, whereas the main function of BAT mitochondria seems to be heat production and the main function of liver mitochondria is

to provide enough ATP for the various liver metabolic functions. The structural differences, as shown by electron microscopy, between mitochondria of various tissues of the rat are obvious, but the qualitative composition of the enzymes related to energy metabolism seems to be relatively similar between various tissues (even BAT mitochondria have all the classical electron transport chain enzymes) and therefore one of the main problems of the study of mitochondrial function is to relate structural, biosynthetic and other possible qualitative or quantitative differences to mitochondrial function.

The third deduction which can be obtained from the turnover studies is that there exists some remarkable discrepancies between the results obtained from various laboratories. The determination of absolute turnover values is complicated by possible influences of undetermined variables such as the nature of the isotope used, its rate of reutilization, the various injection procedures, the age of the animal, the nature of the diet, etc.(*) However, the determination of relative turnover values of various mitochondrial fractions of different tissues made in a same laboratory under controlled conditions is a safer procedure. As this kind of comparative studies was carried out by several laboratories, the general conclusions that the mitochondrion does not turn over as a unit and that mitochondria from different tissues turn over at different rates seem well substantiated.

(*) See Appendix

The diversity of turnover values of various mitochondrial components and the fact that two different genetic systems and two different protein synthesizing systems participate in the biosynthesis of the mitochondrion have led Ashwell and Work (1970) to formulate a dynamic picture of mitochondrial replication:

> Although the mitochondrion itself is an entity with a defined structure we favour the idea that apart from mitochondrial DNA which does indeed replicate by binary fission, there is no such thing as mitochondrial replication. We envisage any one mitochondrial protein as in temporary occupation of a particular binding site and in kinetic equilibrium with other similar but less stable molecules not so bound. The equilibrium between these components would depend upon thermodynamic considerations and the free, less stable, molecules would in turn be in thermodynamic equilibrium with alternative conformations which expose the molecules to hydrolytic cleavage. Each component would then have its own characteristic half-life and would in fact require no mechanism for transport to a specific site but would simply undergo kinetic exchange with a preexisting similar molecule; on this basis, moreover, mitochondrial fusions which appear to be random intracellular events would have no particular relevance to replication. The size of the organelles and the number of DNA molecules per mitochondrion would also vary in a random fashion."

It is very interesting to note that very few studies have been done of the system(s) involved in the turnover of mitochondrial proteins in comparison with the studies made of the mechanisms responsible for protein synthesis. Swift and Hruban (1964) have shown by electron microscopy techniques, several stages of mitochondrial digestion by lysosomes which contain various acid hydrolases and which can theoretically be responsible for the hydrolysis of mitochondrial proteins, DNA, RNA and phospholipids. However, degradation of mitochondria by lysosomes is a mechanism

by which mitochondria would turn over as a unit (Fletcher and Sanadi 1961). This seems not to be the case as discussed previously. It is possible that the phospholipases found in the outer mitochondrial membrane (Waite et al. 1969; Nachbauer and Vignais 1968) or the endopeptidases found in mitochondria (Umana and Dounce 1964) could also participate in the degradation of mitochondrial phospholipids and proteins. In conclusion, the mechanisms and the regulation of mitochondrial protein turnover are not understood.

C. THE RELATION BETWEEN ACCLIMATION TO COLD AND THE BIOGENESIS OF MITOCHONDRIA

Several changes have been observed in mitochondria of various tissues during adaptation to cold, but that there is a relation between these changes and adaptation to cold was only well substantiated for BAT mitochondria. In fact, this tissue undergoes not only a marked hypertrophy and hyperplasia during the first weeks of the adaptation period (Smith and Horwitz 1969), but several biochemical and morphological changes at the mitochondrial level accompany this development. There is a marked increase in the number of mitochondria per cell and in the number of cristae per mitochondrion (Suter 1969; Thomson et al. 1969; Skala et al. 1970), in the total and in the specific activities of several respiratory enzymes such as succinic dehydrogenase and cytochrome oxidase (Barnard et al. 1970; Skala et al. 1970; Jansky et al. 1969) and in the total cytochromes per unit of mitochondrial protein (Skala et al. 1970; Thomson et al. 1969). These changes suggest an increase in BAT mitochondrial protein synthesis during acclimation to cold. Some unspecific changes are also noted in a variety of tissues. These imply an increase in cytochrome c in heart, liver, kidney, lungs, skeletal muscles, and spleen (Klain 1963) in CA rats. Four weeks cold-exposed rats (5°C) showed also an increase in the specific activities of gastrocnemius muscle and liver cytochrome oxidase and succinic dehydro-

genase. Muscle tissue exhibited a greater response in these components than liver tissue (Hannon 1960).

However, similar increases in liver and skeletal muscle cytochrome oxidase were not found in 4 weeks cold-exposed rats (5°C)(Himms-Hagen et al.1972,b).Cold-exposure raised also the coenzyme Q content of rat liver, heart, skeletal muscle and kidney, but not brain (Beyer et al. 1962). Therefore, the main problem with these various mitochondrial changes was to determine if they were correlated or not with the development of NST and if they were mediated or not by the mitochondrial protein synthesizing system.

A specific relation between the capacity for NST (or the capacity to respond to NA) and the biosynthesis of certain mitochondrial proteins might be demonstrated if an inhibition of mitochondrial protein synthesis by specific antibiotics such as oxytetracycline or chloramphenicol were accompanied by a parallel inhibition of the development of NST.

The possibility of a selective inhibitory action of these antibiotics on BAT of the cold-exposed rat was made more likely by studies showing that chloramphenicol appears to affect principally rapidly dividing cells (Firkin and Linnane 1969; Gonzalez-Cadavid et al. 1970). The same antibiotic, which does not inhibit protein synthesis on the cytoribosomes, was shown to inhibit the synthesis of cytochrome oxidase and of the inner mitochondrial membrane of regenerating rat liver cells without affecting that regeneration (Firkin and Linnane, 1969; Kroon and De Vries 1970; Gonzalez-Cadavid et al. 1970). However, it was shown that for "in vivo" studies,

the use of oxytetracycline should be preferred to chloramphenicol because, having also a specific inhibitory action on the mitoribosomes, it is metabolized more slowly than chloramphenicol and produces its effects during a longer period of time (De Vries and Kroon 1970).

In fact, it was shown (Himms-Hagen 1971; Himms-Hagen et al. 1972, b) that repeated intramuscular injection of oxytetracycline to rats exposed at 4°C during 2 weeks prevented the marked increase in the development of the calorigenic response to NA. During this period the growth of the rats and the growth of BAT (as measured by increase in wet weight and total protein content) were not altered by the oxytetracycline treatment, but the normal increase in the specific activity of cytochrome oxidase was strongly inhibited, whereas there was no effect of oxytetracycline on muscle or liver cytochrome oxidase activity (which does not normally increase during acclimation to cold). It was also shown that the effects of oxytetracycline were reversible. Thus, increased synthesis of a small fraction of BAT mitochondrial proteins appears to parallel the development of acclimation to cold. Moreover, if oxytetracycline treatment was continued during the four weeks necessary to acclimate normally a rat to cold, the cytochrome oxidase and the calorigenic response to NA remained both at the initial levels.

These results suggested that:

a) increased BAT mitochondrial protein synthesis appears to accompany the development of the enhanced calorigenic response to NA;

b) as BAT is not the main site of NST, other tissues as skeletal muscle might have been affected by oxytetracycline treatment;

c) the effects of oxytetracycline on other tissues might have been direct or indirect: the inhibition of BAT function by oxytetracycline might have removed an influence of BAT on these tissues.

Thus, the use of oxytetracycline and related antibiotics represents a very useful tool in the elucidation of the mechanisms of NST and of the role of various tissues in adaptation to cold. However, this experimental approach is indirect and should be complemented by a more direct measure of the relations existing between the development of the capacity for NST and the biogenesis of mitochondria. In this thesis, such a direct approach was undertaken. As the mechanism of the switching on of NST and the mechanism of the development of the increased capacity for NST are not understood,and as there still remains some controversy about the sites of NST, it was decided to measure the effects of cold-exposure on mitochondrial protein synthesis and turnover, in various tissues of WA and CA rats. This goal presented two

problems: the choice of the tissues and the choice of the technique. It is obvious from what was said before that BAT, because of its special role in NST, and the skeletal muscles, as the main center of heat production, should be studied. In addition, it was also decided to include a reference organ , the kidney, because it was shown conclusively that this organ is not an important site of NST and finally the liver was chosen for the study as there still remains some controversy about its participation to NST and because it is one of the main sites of metabolic regulation.

The biosynthesis of mitochondrial proteins was studied in these four organs by three different techniques:

- the determination of the half-life of mitochondria and of different mitochondrial protein fractions;
- the "in vivo" rates of AA incorporation in various mitochondrial protein fractions;
- the "in vitro" rates of AA incorporation into mitochondria;

in a study at three different levels:

- a comparison of different mitochondrial protein fractions in the same tissue;
- a comparison of mitochondria of different tissues;
- a comparison of mitochondria of identical tissues in different groups of WA and CA rats.

III. MATERIAL AND METHODS

A. MATERIALS

1. Rats.

Male white rats purchased from Holzman Company were kept at room-temperature for several days after arrival in the laboratory. When their weight was about 150 g, they were divided into warm-exposed and cold-exposed groups and placed into individual cages with free access to water and food. The temperature of the cold room was maintained at $4^{o}C$ and the temperature of the warm room was $25-28^{o}C$. 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.

2. Chemicals

^{14}C-leucine, specific activity 338 or 316 mCi/m mole was purchased from Schwartz Radiochemicals. Pyruvate kinase (E.C. N° 2.7.1.40), cycloheximide, phosphoenolpyruvate (potassium salt), adenosine-5'-triphosphate (disodium salt), Bicine (N,N-bis(2-hydroxyethyl)glycine), Tris (tris-hydroxymethyl-aminomethane), EDTA (Ethylene diamine tetraacetic acid), sodium cholate, sodium deoxycholate, sodium lauryl sulfate were purchased from Sigma Co.

B. METHODS

1) Removal of the organs and determination of their wet weight

The rats were killed at room temperature (25-28°C) by decapitation after determination of their weight. The length of time that the different rats spent in the cold is specified for each experiment under the section Results. No more than 45 seconds elapsed from the removal of the rat from the cold room (4°C) to the decapitation. The liver, the kidneys, the IBAT and the leg skeletal muscles were quickly removed and transferred into their respective ice cold mitochondrial isolation medium. The tissues were allowed to chill before being cleaned, blotted on a filter paper and weighed.

2) Injection of ^{14}C-leucine

The amino acid was injected intravenously at a concentration of 12.5 microcuries/100 g of body weight through a polyethylene cannula which was placed in the tail approximately 2 hours previously while the rats were under ether anesthesia. At the time of the injection, the rats were at the temperature of exposure or acclimation and were conscious. This technique was used in order to avoid any loss of the radioactive amino acid (AA) during the injection procedure and also because a precise timing was required in the experiments where animals were killed only 5 min. after the injection.

3) Isolation of the mitochondria

As one of the principal purposes of this study was to compare mitochondria from different tissues in WA and CA rats, it was

decided to proceed systematically with eight separated mitochondrial isolations in a same experimental day (4 different tissues x 2 experimental groups). Two different methods were used for mitochondrial isolation. Liver, kidneys and BAT mitochondria were isolated essentially as described by Weinbach (1961) and skeletal muscle mitochondria as described by Ernster and Nordenbrand (1967). Skeletal muscle mitochondria were isolated in a medium consisting of 100 mM KCl, 50 mM tris buffer pH 7.4, 1 mM ATP, 5 mM $MgSO_4$, and 1 mM EDTA, and the mitochondria from the three other tissues were isolated in a medium consisting of 0.25 M sucrose, 0.01 M Tris buffer pH 7.4, 1 mM EDTA. The chilled tissues, suspended in their respective isolation media, were cut with scissors until a small mince was obtained. During all the isolation procedures, the temperature of the samples was maintained between 0-4°C. The minced skeletal muscles were homogenized first with a loosely fitting all-glass Potter-Elvehjem homogenizer (that was kept immersed in a vessel containing cracked ice and water) in several short periods of time, for a total time of about 2 min. and then with a tightly fitting homogenizer for the same period of time. The other tissues were homogenized directly with tightly fitting homogenizers for a total period of 1 min. The homogenates were diluted with their respective isolation media to a volume of 10 times the initial weight of the tissues with the exception of BAT which was diluted 40 times its initial weight. The homogenates were centrifuged at 650 g for 10 min. in the SS 34 rotor of the refrigerated Sorvall RC2-B centrifuge. The super-

natants were carefully decanted and centrifuged at 8,500 g for 10 min. (liver and kidney mitochondria). Any layer of fat which rose to the top after the centrifugations was systematically removed. In order to avoid microsomal contamination, the mitochondria were washed four times by resuspension in their respective isolation media and centrifugation at their respective speeds of sedimentation (8,500 g or 14,000 g during 10 min.) (Beattie et al. 1967, a). The different mitochondrial isolations were processed alternately i.e. when muscle and BAT mitochondria were centrifuged, kidney and liver mitochondria were resuspended in the isolation medium and vice versa. In general, eight mitochondrial pellets from two rats were obtained simultaneously in about three to four hours.

4) Fractionation of the mitochondria

Mitochondria were fractionated as described by Beattie et al. (1967, a and 1967, b). The mitochondrial pellets were resuspended and extracted with 5 ml of water at 30°C in a metabolic shaker set at 80 cycles/min and then centrifuged for 10 min. at 27,000 g. the supernatant containing the water-soluble proteins was carefully removed. The remaining pellet was similarly extracted with 5 ml of 0.12 M potassium chloride solution at 30°C during 5 min. After centrifugation at 17,300 g for 10 min. the supernatant yielded the "cytochrome c" fraction. The resulting pellet was then extracted with 5 ml of a 0.6 M potassium chloride solution for 10 min. at 30°C and centrifuged at 27,000 g for 10 min. to remove the "contractile" proteins fraction. The

remaining insoluble proteins were suspended in 5 ml of a 0.25 M sucrose solution and solubilized by the slow addition of sodium cholate (200 mg/mg protein), sodium deoxycholate (100 mg/mg protein) and sodium lauryl sulfate (0.75 mg/mg protein). During the sequential addition of the three detergents, the solutions were frequently mixed by aspiration with a Pasteur pipet equipped with a rubber bulb until an essentially clear solution was obtained. This solution was then treated with a few milligrams of solid sodium dithionate (*) and potassium carbonate, completed to 10 ml with 0.25 M sucrose and brought to 13% saturation with neutralized ammonium sulfate. The solution was left at 4°C overnight to ensure a complete precipitation of the proteins and centrifuged at 34,800 g for 10 min to yield a fraction designated "structural" protein. The resulting supernatant was increased to 50% saturation with ammonium sulfate and the floating yellow-green material was collected with a spatula after centrifugation of the solution at 34,800 g for 15 min. to yield a fraction called "other cytochromes".

In summary, this procedure divides the total mitochondrial population of proteins, on the basis of their solubility, into 5 fractions. It is clear that none of these protein fractions is homogeneous and the names given by several authors (Beattie et al. 1966; 1967, a; 1967, b) do not imply their function. For instance,

* The sodium dithionate is added to keep the cytochromes in a reduced state which ensures a more complete separation of structural protein from the cytochromes (Allman et al. 1967).

it is quite possible that a protein whose only and unique function is "structural" does not exist, the various mitochondrial enzymatic lipoprotein complexes are big enough to have "structural" properties. Recently, this problem has been critically reviewed by MacLennan (1970) who demonstrated conclusively that the notion of a "structural" protein with the properties described by Criddle (1962) has to be abandoned (this fraction contains mainly denaturated proteins derived from the mitochondrial ATPase complex). The same remark holds for the "contractile" protein which may or may not exist (Ohnishi and Ohnishi, 1962; Conover and Barany 1966).

5) Preparation of the proteins for the counting of radioactivity

The different mitochondrial fractions were precipitated with trichloroacetic acid in a final concentration of 5% immediately after they were obtained. The precipitated proteins were washed twice with 5% trichloroacetic acid and once with 5% trichloroacetic acid at 90°C for 5 min. (In the experiments where the rate of AA incorporation into isolated mitochondria was measured, unlabeled leucine was added to all the trichloroacetic acid solutions at a 10 mM concentration). The protein were then dissolved in 2 ml of 0.1 N NaOH containing 10 mM unlabeled leucine and precipitated with trichloroacetic acid at a final concentration of 5%. If this last step was omitted, high background counts were observed. The proteins were extracted twice with ether-ethanol (4:1 v/v) and dissolved in 0.1 N NaOH for the counting of radioactivity and for protein estimation.

6) Protein estimation

Proteins were estimated by the method of Lowry et al. (1951) or by an automated Lowry method as described by Gaunce and D'Iorio (1970). In both methods, bovine serum albumin was used as standard.

7) Counting procedures

Small aliquots (0.2 ml) of the protein dissolved in 0.1 N NaOH were counted in 10 ml of Bray's solution (Bray 1960), or alternately 1 ml of the dissolved proteins were mixed with 10 ml of Beckman's BBS-3 Ready Solv solution containing 5 g/l of 2,5-diphenyloxazole(PPO). The samples were counted at room-temperature in a Beckman LS-250 liquid scintillation counter. All the counts/min (CPM) were corrected for quenching and converted to disintegrations/min (DPM). Counting efficiency was in general 86%.

8) Amino acid incorporation by isolated mitochondria

All glassware, centrifuge tubes and solutions necessary for the isolation and incubation of the mitochondria were sterilized by autoclaving them for 20 min. at 121°C at a pressure of 20 lbs/square inch. Only heat degradable products such as ATP, phosphoenolpyruvate or enzymes were added to ice cold sterile solutions. BAT and liver mitochondria were isolated as described earlier in a sterile medium containing 250 mM sucrose, 1 mM EDTA and 30 mM bicine buffer pH 7.4 and skeletal muscle mitochondria were isolated in a medium containing 100 mM KCl, 5mM $MgSO_4$, 1mM EDTA and 30 mM bicine buffer pH 7.4. The final pellet of the isolated

and washed mitochondria was suspended by gentle hand homogenization at a concentration of about 5-10 mg/ml in a medium containing:

- 10 mM KH_2PO_4
- 154 mM KCl
- 10 mM $MgCl_2$
- 1 mM EDTA (di Na salt)
- a mixture of 19 natural AA (except leucine) 5 microM each
- 30 mM bicine. The final pH was adjusted to 7.4

0.1 ml of the resulting suspension was added to 0.9 ml of the incubation medium at 0^oC. The incubation medium contained:

- 10 mM KH_2PO_4
- 154 mM KCl
- 10 mM $MgCl_2$
- 1 mM EDTA (di Na salt)
- a mixture of 19 natural AA (except leucine) 5 microM each
- 5 mM phosphoenolpyruvate (potassium salt)
- ^{14}C- leucine, 1 microcurie/ml; specific activity 316 mCi/m Mole
- pyruvate kinase (E.C. N° 2.7.1.40): 0.1 mg/ml; specific activity 380 units/mg protein; unit definition: one unit will convert 1.0 mM of phosphoenolpyruvate to pyruvate per min. at pH 7.6 at 37^oC.
- cycloheximide 0.5 mg/ml
- adenosine 5'-triphosphate was added at several different concentrations: 0 mM, 0.2 mM, 0.5 mM 1 mM, 2 mM, 5 mM and 10 mM
- 30 mM bicine. The final pH of each solution was adjusted to 7.4 after addition of all the components

After a preincubation period of 6 min. at 0°C, the reaction was started by transferring the mitochondria in sterile flat-bottomed flasks (2.2 cm diameter - loosely sealed with a plastic cap), shaking at 80 cycles/min at 37°C in a metabolic shaker. The reaction was stopped 15 min. later by the addition of 1 ml of ice cold 10% trichloroacetic acid containing 10 mM leucine and by the simultaneous transfer of the flasks on cracked ice.

9) Estimation of leucine concentration in various tissues of the rat

After the wet weights of the tissues were determined as described above, they were cut in small pieces and homogenized directly in 7 % sulfosalicylic acid. Blood was collected immediately after decapitation in heparinized beakers on ice and subsequently centrifuged. One sample of plasma (2 ml) was deproteinized with 2 ml of 14% sulfosalicylic acid. All samples were extracted three times with 7% sulfosalicylic acid and the different extracts were mixed and diluted to a known volume. The concentration of leucine was determined on the Beckman AA autoanalyzer model 120B according to the method of Spackman et al. (1958). One ml of the sample was applied on the top of a column of 0.9x50 cm containing AA - 15 resin (Beckman) equilibrated at pH 3.28 with a 0.2 N citrate buffer. The AA were eluted with a 0.2 N citrate buffer pH 4.25. The quantity of leucine was determined after elution from the column and subsequent reaction with ninhydrin from the area of the peak traced by the recorder and a color constant which was obtained by passing a known quantity of leucine through the analyzer.

The general formula used was:

$$\text{Micromoles of leucine} = \frac{H \times W}{C}$$

H = height of peak

W = width in dots

C = color constant (C=22.1 for leucine)

10) Methods of calculation and statistical evaluation of the data

The half-lives ($t\frac{1}{2}$) of the different mitochondrial protein fractions were calculated from the regression coefficient (b) which was obtained by regression analysis by the least-square method. The analysis and the determination of the equation of the logarithmic regression line was calculated on a Wang calculator Model 380, essentially as described in the program No. Cal 360 - STAT - 5 of the Wang Program Library. The equation of the regression line $y = a+bx$, where a is the intercept on y-axis at $x = 0$, and b is the slope (b is also known as the regression coefficient) was calculated according to the equations:

$$b = \frac{\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{N}}{\Sigma x^2 - \frac{(\Sigma x)^2}{N}}$$

and

$$a = \frac{\Sigma y - b\Sigma x}{N}$$

where N = total number of individual determinations
x = days
y = log of the specific activity

The standard error of the mean, the 95% confidence intervals and the "t" test of significance between the regression coefficients were calculated according to Snedecor (1955) and Goldstein (1964).

IV. RESULTS

The following scheme has been adopted for the presentation of the results of each experiment: a) Purpose of the experiment; b) Principle of the technique; c) Description of the experiment; d) Results; e) Discussion (specific to the experiment). The presentation of the results will be followed by a general discussion and conclusion.

1. Determination of the half-lives of different mitochondrial protein fractions of liver, kidney, interscapular brown adipose tissue and skeletal muscles of WA and CA rats.

a) Purpose of the experiment: to see if in CA rats, there are specific changes in the half-lives of various mitochondrial protein fractions in tissues known to be important sites of NST (BAT and skeletal muscle) and in other tissues where NST does not take place, or where there is no increased capacity for making NST during acclimation to cold (kidney and liver).

b) Principle of the technique: the half-lives of the mitochondrial protein fractions were determined by labelling the proteins with ^{14}C-leucine and measuring the rate of disappearance of the label. It should be noted that this technique does not distinguish between the mitoribosomal or the cytoribosomal origin of the mitochondrial proteins which turn over.

c) <u>Description of the experiment</u>: 34 rats were divided in two equal groups of WA and CA rats. The rats had lived for approximately 2 months at their acclimation temperature when this experiment was started. Body weights of the rats at this time were, WA rats: 364 $\pm$ 7.5 g (17 rats); CA rats: 301 $\pm$ 8.5 g (17 rats). Two rats (one from each experimental group) were injected intravenously with ^{14}C-leucine (12.5 microcuries/100 g body weight) as described under "Material and Methods" and remained at the temperature of acclimation until they were killed. 5 rats from each experimental group were killed after 2 days, 3 after 7 days, 4 after 10 days and 5 after 14 days. The mitochondria of liver, kidney, IBAT and skeletal muscle were isolated, fractionated and processed for counting as described under "Material and Methods". Protein and ^{14}C-leucine content were measured in each fraction and the specific activities of the various fractions obtained from the four tissues of WA and CA rats were plotted against time on a semi-logarithmic scale. The equation of the regression line was obtained by regression analysis (cf. Material and Methods).

d) <u>Results</u>: the half-lives calculated from the regression coefficients and a statistical evaluation of the data are reported in Table 4 a and 4 b (cf." Material and Methods"). For better comparison, the values of the half-lives of different mitochondrial fractions are also plotted in figure 12.

- There is a significant decrease in the half-lives of BAT and skeletal muscle mitochondria in CA rats living in the cold

Table 4 a and 4 b: Half-lives of various mitochondrial protein fractions of liver, kidney, BAT and skeletal muscle of CA rats. These tables give the regression coefficients (-b), the number of individual determinations (indicated between brackets), the standard errors of the mean ($\pm$ SE), the 95% confidence intervals (95%) and the half-lives ($t\frac{1}{2}$) calculated from the regression coefficients of the different mitochondrial fractions. Probability values (P) that two corresponding slopes of WA and CA rats were significantly different were calculated by the t test. The calculation methods and the statistical evaluation of the data were described under "Material and Methods". The differences between the WA rats and the CA rats are significant for skeletal muscle total mitochondria ($P < 0.02$), contractile proteins ($P < 0.01$), and structural proteins ($P < 0.01$). Differences are also significant for BAT total mitochondria ($P < 0.01$) and structural proteins ($P < 0.01$). No other differences are significant at the 5% level (NS).

TABLE 4A

	LIVER - WA GROUP					LIVER - CA GROUP			
Fraction	-b	± SE	95%	t½	P	-b	± SE	95%	t½
Total	.0428 (16)	.0037	.0079	7.03	NS	.0386(17)	.0046	.0097	7.79
H_2O-Sol.	.0374 (17)	.0042	.0091	8.05	NS	.0400(17)	.0055	.0118	7.51
Cyto. C.	.0491 (17)	.0087	.0185	6.13	NS	.0492(17)	.0059	.0125	6.11
Contractile proteins	.0508 (16)	.0069	.0148	5.92	NS	.0456(15)	.0053	.0115	6.63
Struct.	.0440 (16)	.0037	.0080	6.84	NS	.0460(17)	.0047	.0101	6.65
Other Cytochr.	.0483 (16)	.0048	.0103	6.23	NS	.0591(17)	.0070	.0150	5.10

	KIDNEY - WA GROUP					KIDNEY - CA GROUP			
Total	.0413 (16)	.0037	.0080	7.29	NS	.0452(17)	.0051	.0109	6.65
H_2O-Sol.	.0514 (16)	.0050	.0108	5.86	NS	.0573(15)	.0031	.0067	5.25
Cyto. C	.0433 (14)	.0046	.0100	6.95	NS	.0441(14)	.0099	.0216	6.82
Contractile proteins	.0282 (13)	.0047	.0104	10.70	NS	.0338(13)	.0098	.0216	8.89
Struct.	.0312 (15)	.0039	.0085	9.65	NS	.0342(17)	.0048	.0102	8.79
Other Cytochr.	.0298 (16)	.0036	.0079	10.09	NS	.0416(16)	.0045	.0097	7.23

TABLE 4B

Fraction	SKELETAL MUSCLE - WA GROUP					SKELETAL MUSCLE - CA GROUP			
	-b	± SE	95%	$t_{\frac{1}{2}}$	P	-b	± SE	95%	$t_{\frac{1}{2}}$
Total	.0133(17)	.0043	.0093	22.65	.02	.0258(17)	.0034	.0067	11.68
H_2O-Sol.	.0197(11)	.0025	.0056	15.30	NS	.0272(12)	.0060	.0152	11.06
Contractile Proteins	.0140(16)	.0066	.0140	21.57	.01	.0372(17)	.0054	.0114	8.09
Struct.	.0097(17)	.0045	.0095	30.90	.01	.0225(17)	.0032	.0069	13.39
Other Cytochr.	.0139(15)	.0048	.0103	21.69	NS	.0178(17)	.0053	.0113	16.88

Fraction	BAT - WA GROUP					BAT - CA GROUP			
	-b	± SE	95%	$t_{\frac{1}{2}}$	P	-b	± SE	95%	$t_{\frac{1}{2}}$
Total	.0242(17)	.0041	.0088	12.49	.01	.0403(17)	.0040	.0085	7.47
H_2O-Sol.	.0235(9)	.0064	.0080	12.81	NS	.0345(17)	.0071	.0152	8.74
Struct.	.0240(17)	.0044	.0093	12.23	.01	.0407(17)	.0042	.0090	7.40
Other Cytochr.	.0375(14)	.0067	.0146	8.03	NS	.0250(17)	.0070	.0149	12.04

(Fig. 11 c and d; Table 4 b; Fig. 12). This decrease occurs in the relatively insoluble "contractile" and "structural" protein fractions (Fig. 11 m, q, p; Table 4 b; Fig. 12). In contrast, no change in the half-lives of various kidney or liver protein fractions was observed (Fig. 11 a, b, e, f, i, j, k, l, n, o, r, s; Table 4 a; Fig. 12).

- Figures 11 a to 11 u show that the various regression lines of mitochondrial proteins of CA rats are drawn systematically at lower levels of specific activity than the regression lines of corresponding fractions of WA rats, even for the various liver or kidney mitochondrial protein fractions where no change in turnover was detected. This suggests that there is an apparent inhibition of leucine incorporation in various mitochondrial protein fractions in the CA rats living in the cold.

e) Discussion:(*) the specific decrease in the half-life of certain mitochondrial proteins in tissues where NST is known to take place (skeletal muscle and BAT) and not in other tissues which are not important sites of NST (liver and kidney) suggests that it might be associated with the development of the increased capacity for NST or with the operation of the heat producing metabolic processes during NST. This decrease in the half-lives of BAT and skeletal muscle mitochondria is associated mainly with the insoluble mitochondrial protein fractions which are believed to be synthesized at least partially by the mitochondrial protein synthesizing system (cf. introduction). Depocas (1966) has previously shown

(*) See Appendix

Figures 11 a to 11 u: Regression lines of various mitochondrial protein fractions of liver, kidney, BAT and skeletal muscle of WA and CA rats. The equation of each regression line was calculated by regression analysis by the method of least squares (cf. Material and Methods) using the number of individual determinations reported in Tables 4 a and 4 b. The points represent the mean of the specific activities and the vertical lines the standard errors of the mean of various groups of rats (5 rats were killed after 2 days, 3 after 7 days, 4 after 10 days and 5 after 14 days). The open points represent WA rats and the black points CA rats.

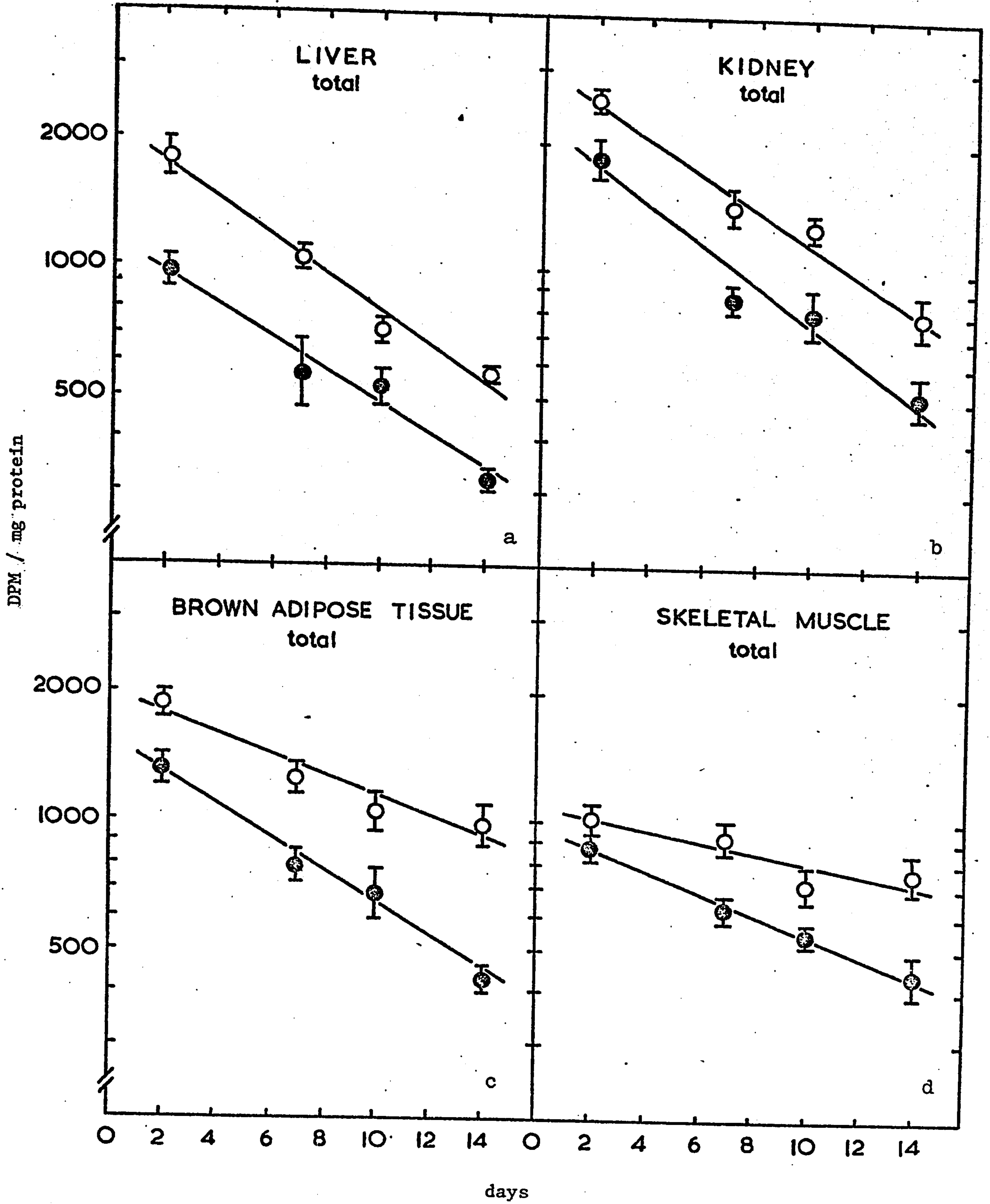


Figure 11

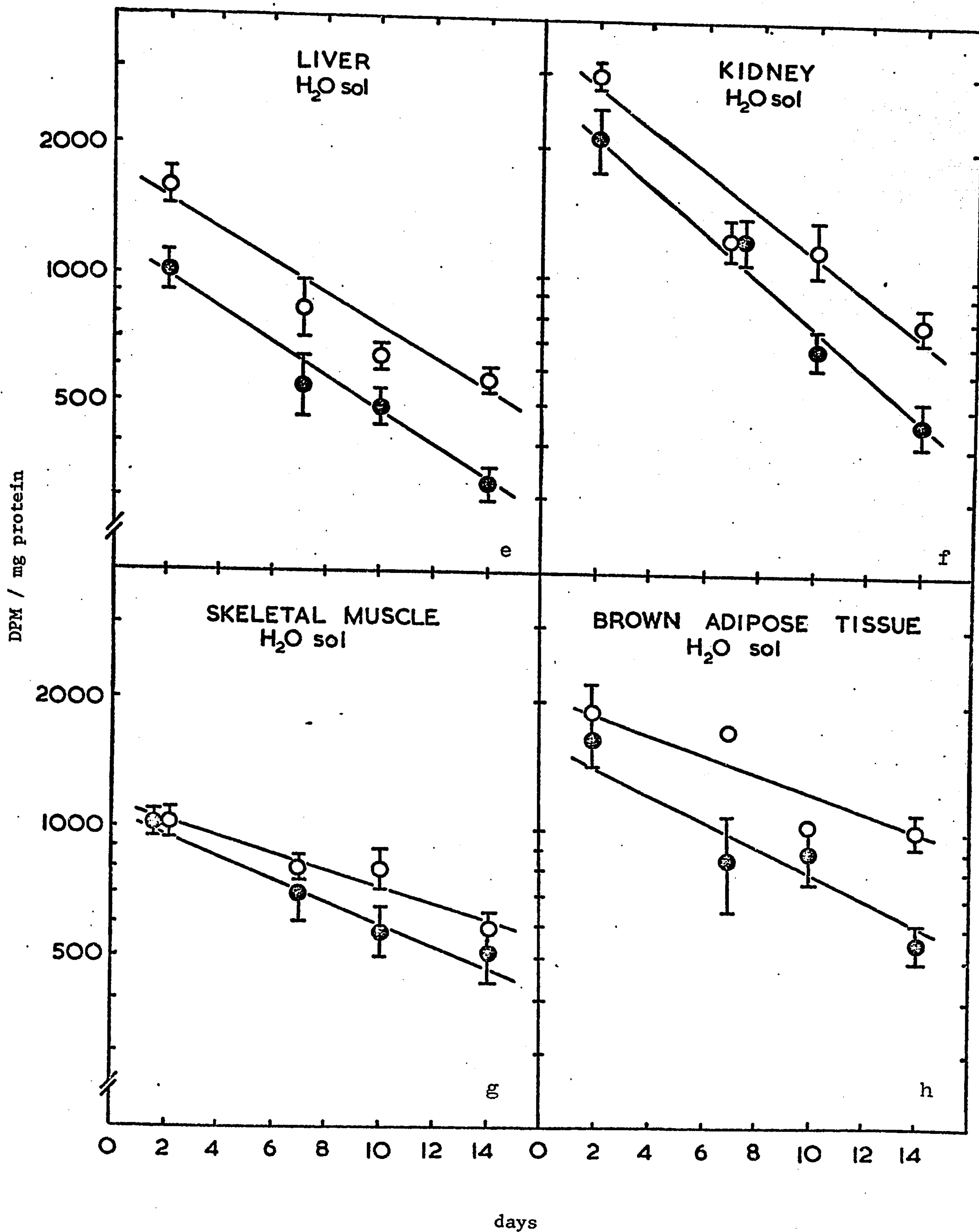


Figure 11

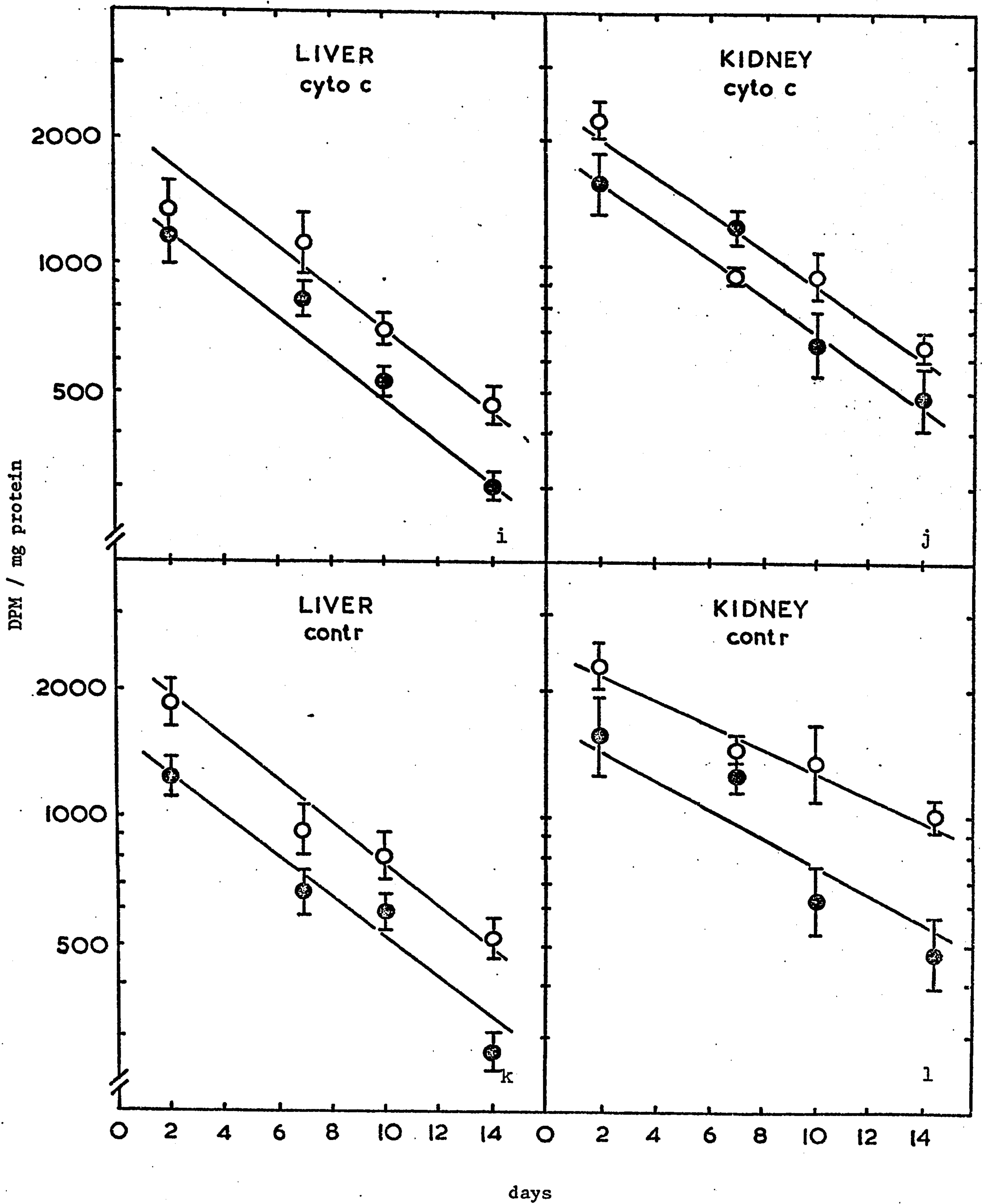
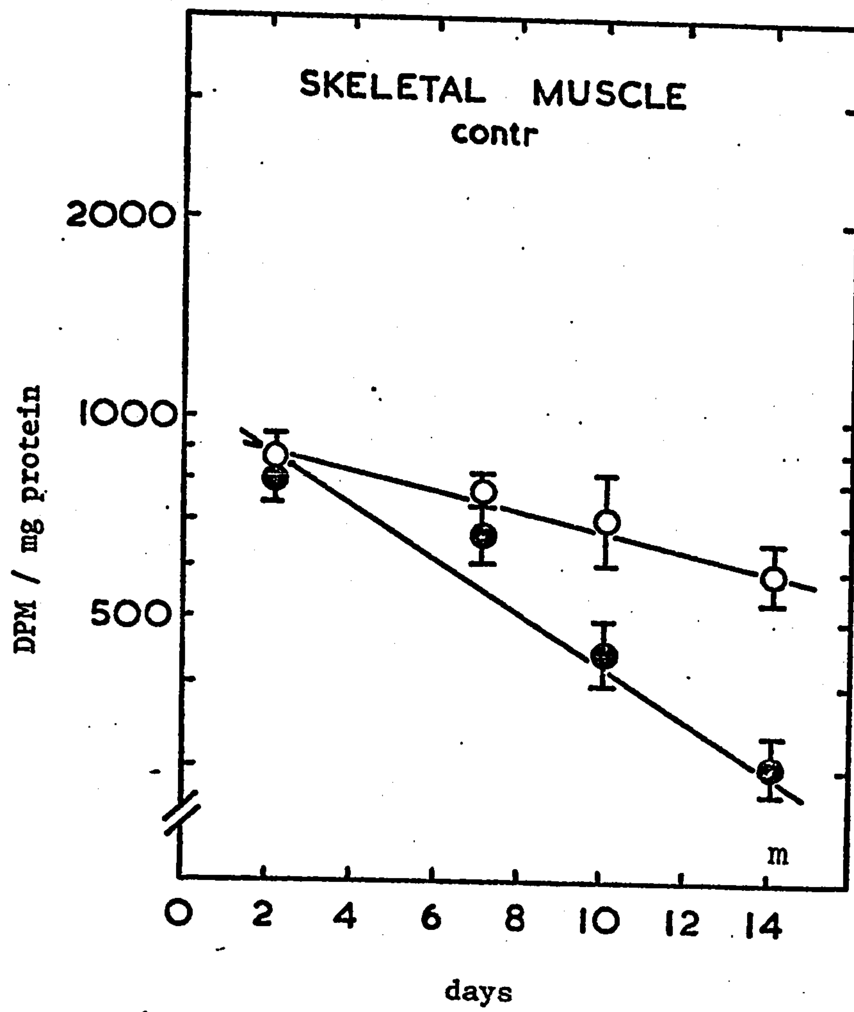


Figure 11



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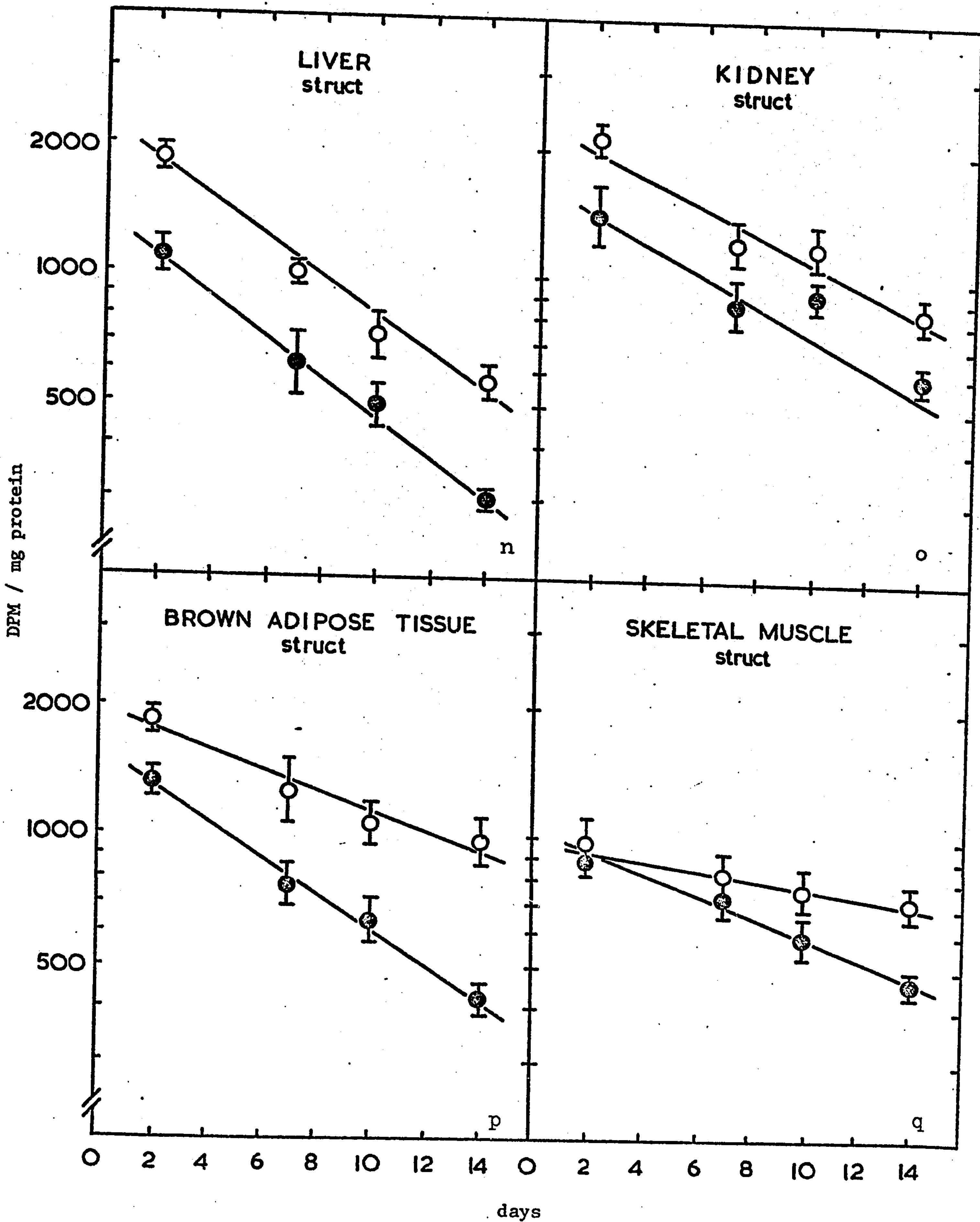

Figure 11

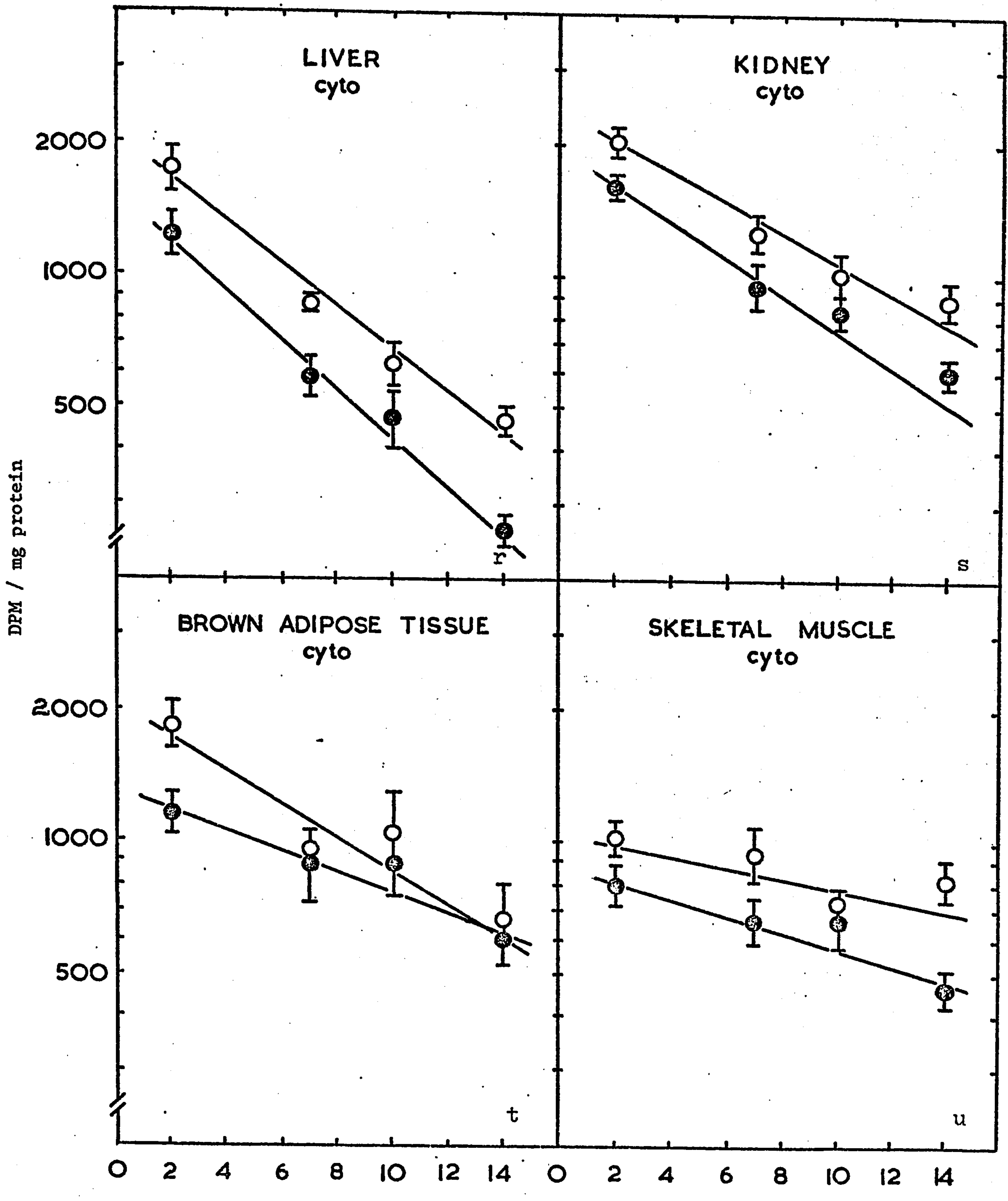

Figure 11

Figure 12: Half-lives of various mitochondrial protein fractions of liver, kidney, BAT and skeletal muscle of WA and CA rats. The half-life (in days) is given for total mitochondrial proteins in tissues of WA (open bars) and CA (black bars) rats.

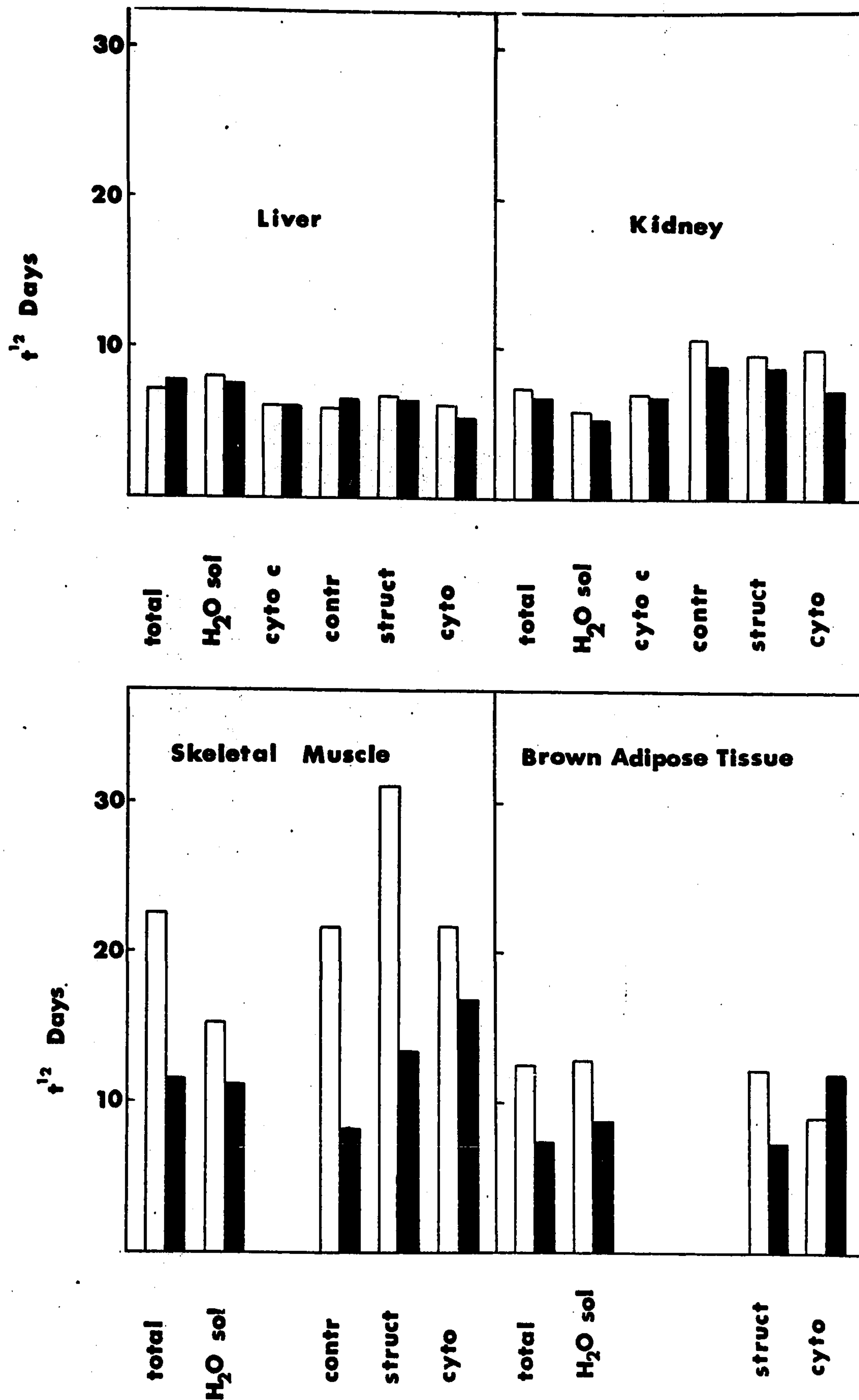

Figure 12

that purified cytochrome c isolated directly from skeletal muscle tissue does not turn over more rapidly in CA rats. Cytochrome c, like the majority of the water-soluble proteins is synthesized on the cytoribosomes (Beattie 1967, a; 1969; Clark-Walker and Linnane 1968; Gonzales-Cadavid and Campbell 1967; Kadenbach 1970). Not enough cytochrome c fraction material isolated from skeletal muscle mitochondria could be obtained in all the experiments for estimation of radioactivity. This fraction accounts for only 5% of the total mitochondrial proteins (*). However, we did not observe a significant difference in the water-soluble protein fraction between CA and WA rats where cytochrome c is certainly also dissolved (Beattie 1966).

The half-lives observed for the various mitochondrial proteins of liver and kidney agree well with previous estimates and the lack of effect of cold-acclimation on the turnover of total liver mitochondrial proteins agrees also with the findings of Lusena and Depocas (See Table 3).

A similar change in the half-lives of certain mitochondrial fractions and not in others was recently reported for liver mitochondria of thyroidectomized rats by Katyare et al.(1972) who observed

(*) Because of small quantities of starting material, Beattie et al. (1967, c) mixed the "cytochrome c" fraction with the "contractile" protein fraction of kidney obtained from mitochondria in a 0.6 M KCl soluble fraction and divided brain mitochondria simply into water-soluble and water-insoluble proteins. In this experiment it was decided to avoid the mixing of several fractions even for the BAT mitochondria, in order to have more pure and comparable fractions. The fraction which was in all tissues more abundant was the "structural" protein fraction which represented in general 30-40% of the total mitochondrial proteins.

an increase of 20-40% in turnover of "contractile" proteins, "structural" proteins and "other cytochromes" fractions with no change in the turnover of water-soluble proteins and cytochrome c fractions; the authors suggested a role of thyroid hormones in the synchrony of turnover of mitochondrial proteins in the euthyroid state.

The results of Katyare et al. (1972) and the changes in the half-lives of certain specific mitochondrial protein fractions of skeletal muscle and BAT of CA rats reported here, suggest, as it was already discussed in the introduction, that it is unlikely that mitochondria turn over as a unit.

It has to be noted that changes in turnover values of mitochondrial protein fractions do not distinguish between a change in the rate of synthesis and degradation of one or more proteins in the group and a change in the amount of one or more proteins in the group. Another possibility is that these changes in the half-lives are due to changes in the population of different mitochondria. In this context it could be mentioned that recently Gross (1970) showed, in studies of the effect of thyroxine on the turnover of liver mitochondrial DNA in thyroidectomized rats, by the use of double labeling techniques, that there was an increase in turnover of liver mitochondrial DNA after the administration of thyroid hormone to thyroidectomized rats. Gross suggested that there are in liver two populations of mitochondria, one synthesized at high levels of thyroid hormone, the other at low levels. Moreover, different types of mitochondria which could apparently be related to the physical state of heart muscle or

skeletal muscle were recently isolated by Hülsmann (1970) and by Bullock et al.(1971).The hypothesis that there are various types of mitochondria in a given tissue and that the proportions of these mitochondria could change under the influence of physiological changes needs further confirmation.

The apparent inhibition of leucine incorporation in mitochondrial fractions of different tissues (Figures 11 a to 11 u) could in principle be interpreted as a general inhibition of mitochondrial protein synthesis in the CA rat. Similar inhibitions were observed by Lusena and Depocas (1967) in turnover studies of liver mitochondria and by Kim (1967) in direct studies of "in vivo" AA incorporation in heart, spleen, liver, kidney and brain mitochondria. However, it seems that it is unlikely that there is such a general and unspecific inhibition of mitochondrial protein synthesis in the CA rat. The reason is logical: The results presented here and the studies of Lusena and Depocas (1967) have both demonstrated that there is no change in the half-life of liver mitochondria of CA rats which is about one week. If an inhibition of only 20-30% of mitochondrial protein synthesis occurs in these tissues, then after 3 to 4 weeks there should be nearly no mitochondria in the livers of CA rats, which is certainly not observed. This problem was further analyzed by a comparison of "in vivo" and "in vitro" studies of AA incorporation in various mitochondrial protein fractions as well as by the determination of the concentration of free leucine in plasma and various other tissues. (See pages 112 and 125). (*)

(*) See Appendix

2. Determination of the "in vivo" rate of amino acid incorporation into various mitochondrial protein fractions of liver, brown adipose tissue and skeletal muscles of WA, CA and cold-exposed rats.

a) Purpose of the experiment: this study is in reality the complement of the preceding experiment, where it was found that there was aa decrease in the half-life of certain insoluble mitochondrial proteins in skeletal muscle and BAT of CA rats. As mitochondrial protein metabolism can be regulated at the level of the degradation or synthesis of proteins, it was decided to measure the synthesis of mitochondrial proteins in the same fractions (except kidney) as those in which their degradation was measured.

b) Principle of the technique: ^{14}C-leucine incorporation in various mitochondrial protein fractions was measured "in vivo". In principle, this technique cannot distinguish absolutely between the cytoplasmic or the mitochondrial origin of the protein participating in mitochondrial structure. However, as shown by Beattie et al. (1966), if the rats are killed at short intervals of time after the intravenous injection of the label, the specific activity of the water-soluble proteins and cytochrome c fraction (made probably on the cytoribosomes) is lower than the specific activity of the more insoluble proteins. These kinetic studies suggested that the water-soluble proteins are

synthesized at an extramitochondrial site and transferred into the mitochondria in a subsequent step. After further studies on the biogenesis of mitochondrial protein components in rat liver slices, Beattie (1968) suggested that the biogenesis of mitochondria involves the initial synthesis of certain insoluble proteins by the mitochondria and the subsequent integration of soluble proteins synthesized outside the mitochondrion. It was therefore decided to kill the rats at short intervals of time after the injection of the label in order to measure mitochondrial protein synthesis more specifically.

c) <u>Description of the experiment</u>: thirty rats were divided in several WA, CA and cold-exposed (CE) groups. The number of rats in each group, the time of cold-exposure and their average weights were:

GROUP	TIME IN COLD	NUMBER OF RATS	WEIGHT (in grams)
1. Young WA rats	0 hours	5	174 ± 11.5
2. Young CE rats	12 "	4	171 ± 3.6
3. Young CE rats	3 days	5	170 ± 6.6
4. Young CE rats	14 "	3	251 ± 24.9
5. WA rats (control of group 4)	-	3	291 ± 9.8
6. CA rats	6-8 weeks	5	294 ± 14.3
7. WA rats (control of group 6)	-	5	357 ± 13.2

The rats were injected with 12.5 microcuries/100 g of body weight, as described under "Material and Methods". Exactly 4 min. after the injection, the rats received another intravenous injection of 18.75 micromoles/100 g of body weight of unlabelled leucine and were killed by decapitation one minute after (exactly 5 min. after the initial injection of the label). The animals were bled during 40 seconds, and liver, IBAT and skeletal muscles were removed, transferred to their ice cold isolation media at 6 min. 15 sec. (liver), 7 min. (BAT) and 8 min. (skeletal muscle), and allowed to chill. The mitochondria were isolated, fractionated and treated for protein and radioactivity estimation as described under "Material and Methods". The activity specific of the various mitochondrial protein fractions in liver, BAT and skeletal muscle at different times of cold-exposure or after cold-acclimation are shown in figures 13, 14 and 15. The same results calculated as percentages of the corresponding control WA group are reported for the three tissues in figures 16, 17, 18.

d) Results: The water-soluble proteins and cytochrome c fraction of liver mitochondria and the cytochrome c and "contractile" protein fractions of BAT mitochondria in WA or CA rats have lower specific activities than the relatively insoluble "structural" mitochondrial protein fraction (Figures 13, 14). Thus according to Beattie (1966) the majority of the proteins in the relatively soluble fractions could be synthesized on the cytoribosomes, and subsequently transferred and incorporated in the

Figures 13, 14 and 15: Specific activities of various mitochondrial protein fractions of liver (Fig. 13), BAT (Fig. 14) and skeletal muscle (Fig. 15) in WA, CA and cold-exposed(CE)rats killed 5 min. after the intravenous injection of ^{14}C-leucine. The numbers under the bars refer to different times of cold-exposure, 1 : 0 hours; 2 : 12 hours; 3 : 3 days; 4 : 14 days; 5 : 6-8 weeks. Open bars represent WA rats and black bars CA or CE rats. The vertical lines represent the standard errors of the mean.

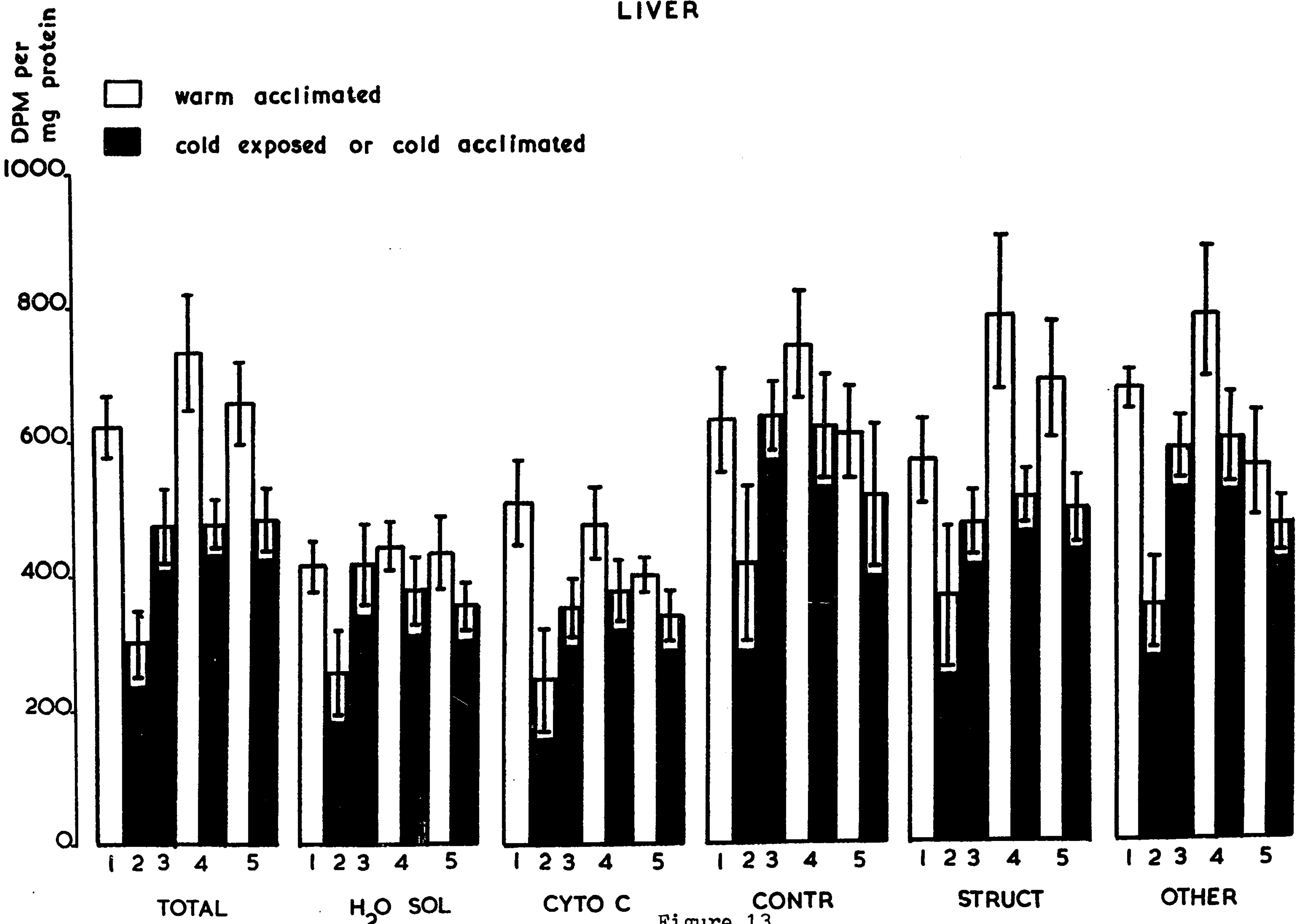

Figure 13

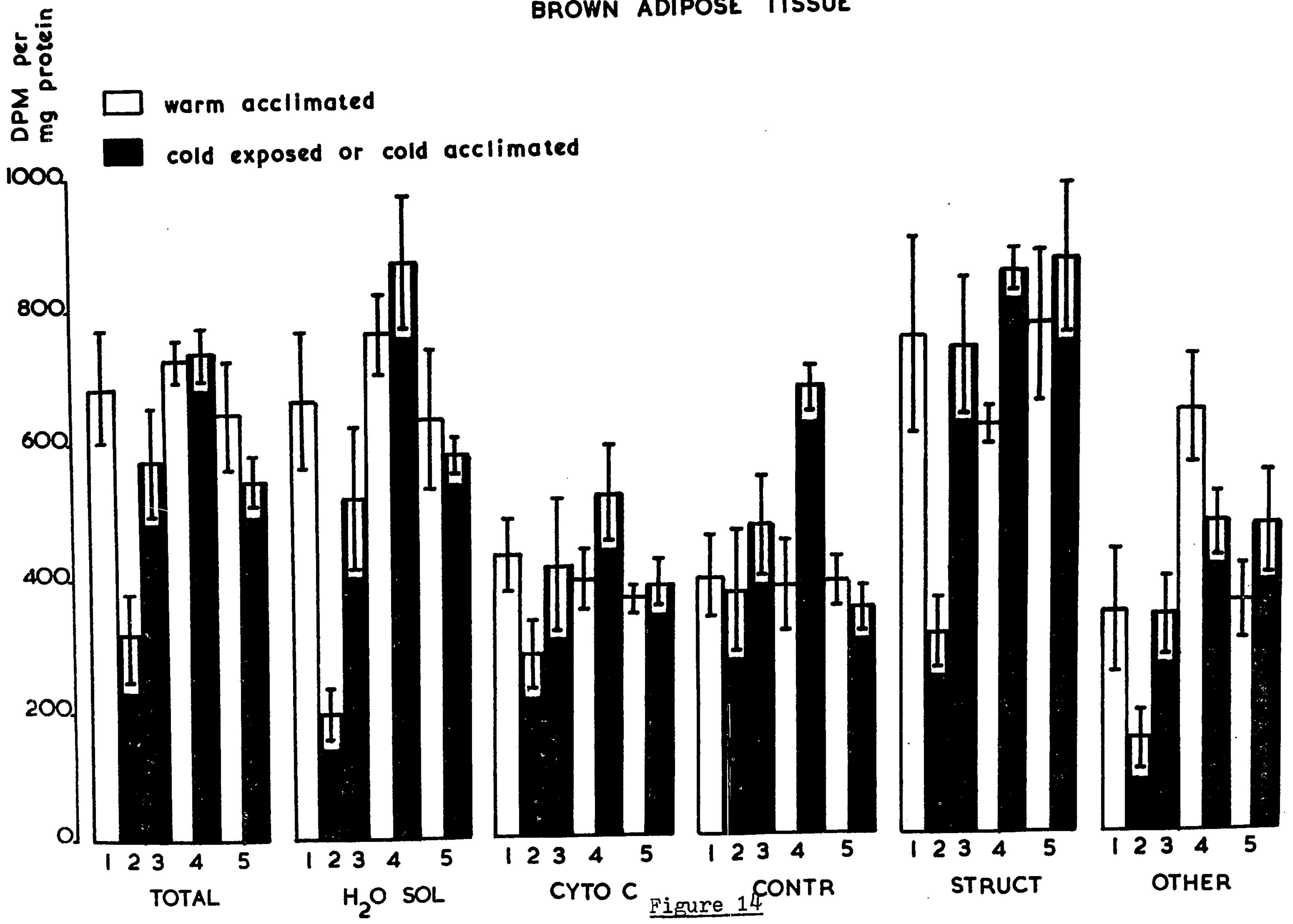

Figure 14

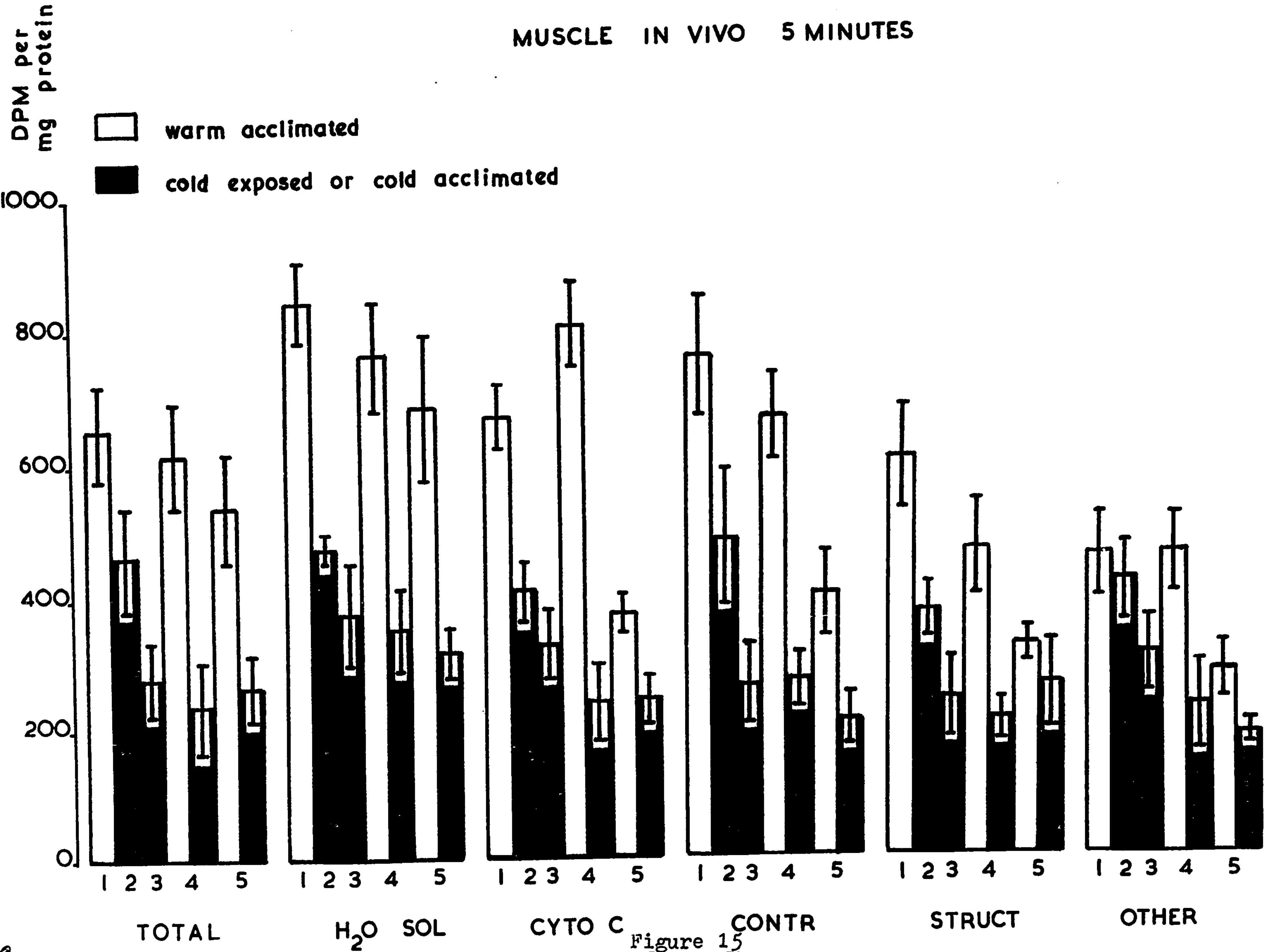

Figure 15

Figures 16, 17 and 18: Specific activities of various mitochondrial protein fractions of liver (Fig. 16), BAT (Fig. 17) and skeletal muscle (Fig. 18) in cold-exposed or CA rats killed 5 min. after the intravenous injection of ^{14}C-leucine. The specific activities were calculated as percentages of the corresponding control WA groups.

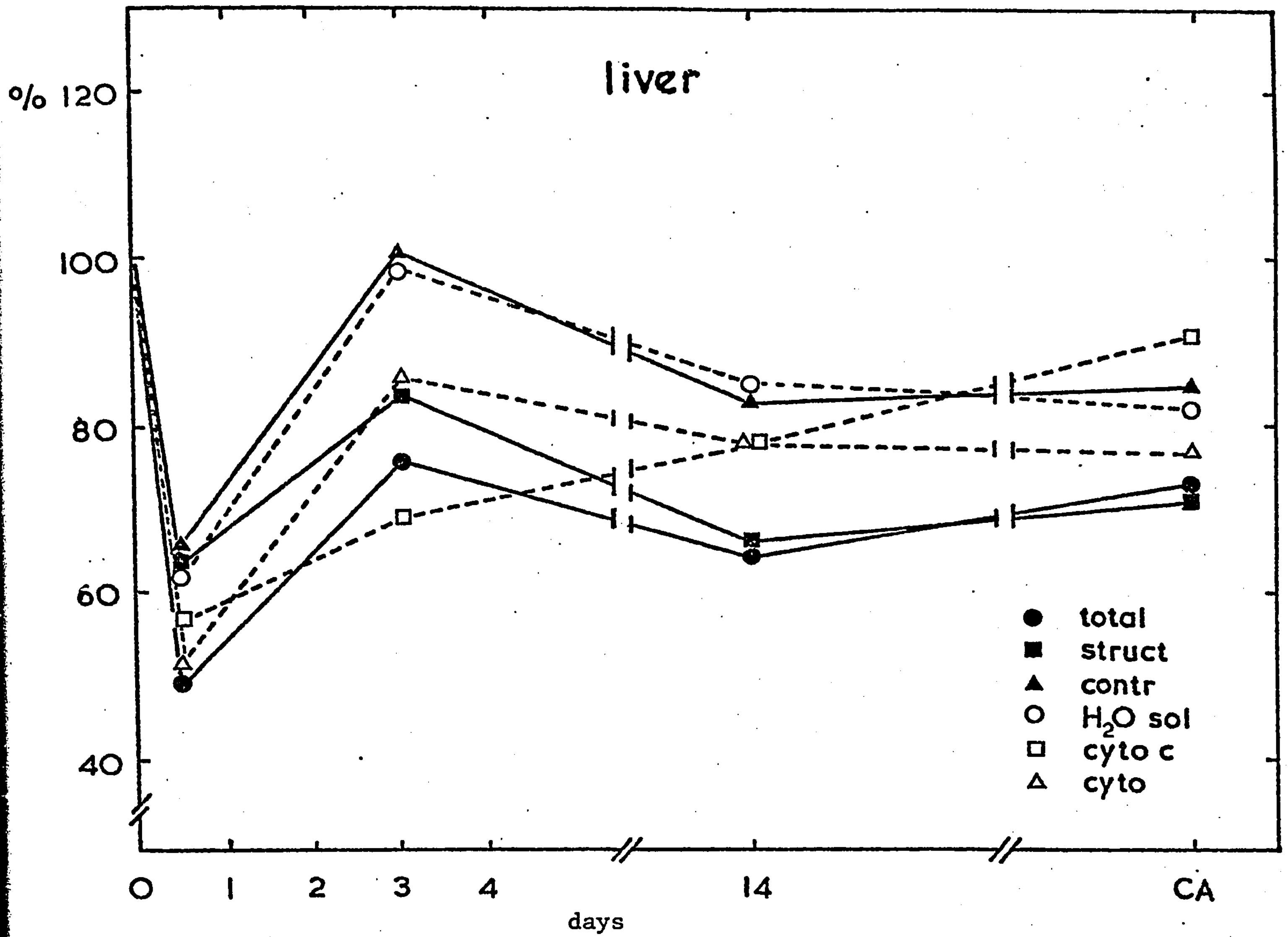

Figure 16

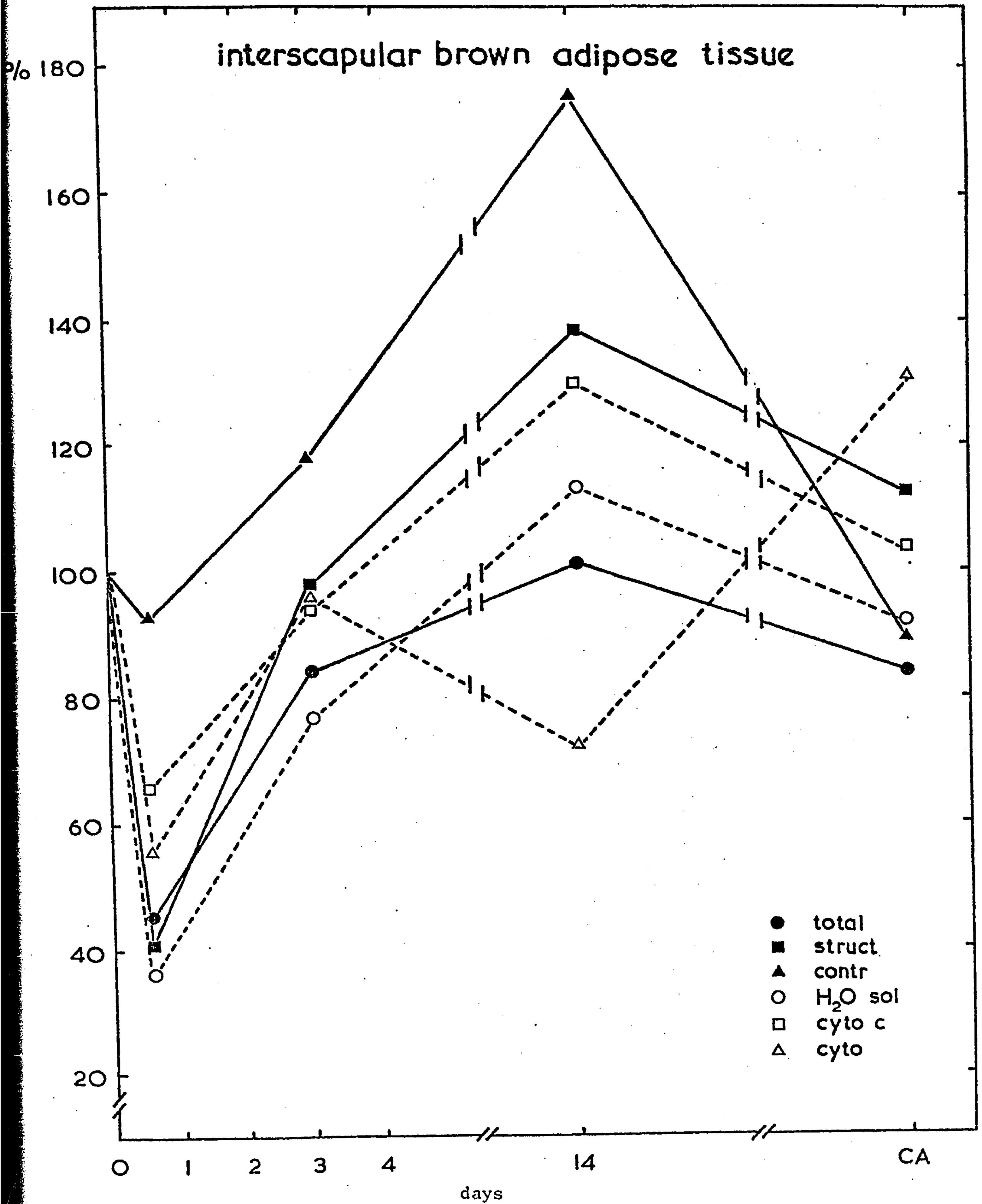

Figure 17

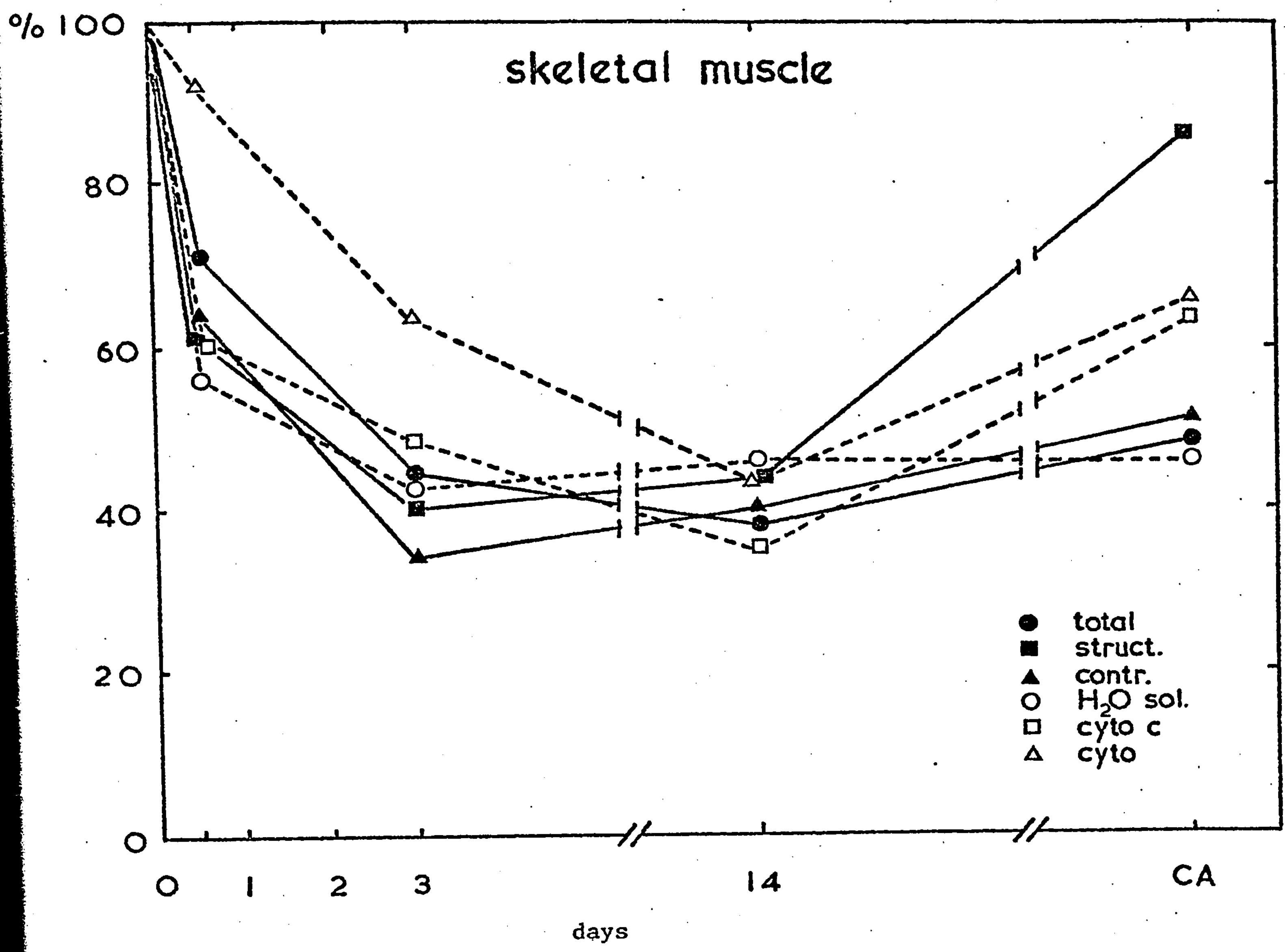

Figure 18

mitochondrial structure. On the contrary, the specific activity of the water-soluble and cytochrome c fraction of skeletal muscle mitochondria is even higher than the specific activity of the more insoluble fractions (Fig. 15). As it is unlikely that the cytoplasmic or mitochondrial origin of the proteins participating in the structure of muscle mitochondria is different from the mitochondria of other tissues, it is possible that the basic assumption underlying the principle of the technique (i.e. that mitochondrial AA incorporation after 5 min. represents mitochondrial protein synthesis) is not justified for muscle mitochondria.

The comparison of AA incorporation in liver, BAT and skeletal muscle mitochondria shows that:

1) Liver: After 12 hours of exposure to cold there is an initial inhibition of leucine incorporation in various mitochondrial fractions which is followed by a general increase of incorporation to slightly lower levels than the control WA group. No further change was noted during all the time necessary to acclimate a rat to cold.

2) Brown adipose tissue: (Fig. 17) A similar inhibition to that observed with liver mitochondria occurs after 12 hours of cold-exposure but thereafter there is a marked increase of leucine incorporation which peaks at 14 days of cold-exposure and regresses during the last weeks of acclimation to cold. This increase occurs principally in the "contractile" and "structural" proteins but occurs also to a smaller extent in all the other

fractions of CA rats. Leucine incorporation in BAT mitochondria is similar in WA and CA rats with the exception of the "other cytochromes" fraction where it is higher in the CA group.

3) Muscle: (Fig. 18) As in the two other tissues a marked initial inhibition of leucine incorporation was observed after 12 hours of cold-exposure. However, that inhibition persisted during the first 2 weeks of exposure to cold and only during the last weeks of acclimation to cold, leucine incorporation increased in various fractions of muscle mitochondria.

e) Discussion(*): As was already suggested during the mitochondrial protein turnover experiments (See Fig. 12), there is in fact an apparent inhibition of leucine incorporation in various liver and skeletal muscle mitochondrial fractions in the CA rat (See figures 16 and 18). This inhibition occurs also during acclimation to cold with the only exception of BAT mitochondria where there is a remarkable increase of leucine incorporation during the first 2 weeks of cold-exposure (See Fig. 17). It is interesting to note that there is a relatively unspecific inhibition of leucine incorporation after 12 hours of cold-exposure in all the three tissues investigated, but during the following 2 weeks the patterns diverge: leucine incorporation in liver mitochondria returns to nearly normal levels, in muscle mitochondria it continues to decrease slightly, whereas in BAT mitochondria it increases. Moreover, during the last weeks of acclimation to cold, leucine incorporation does not change in liver mitochondria, whereas it decreases in BAT mitochondria and in-

(*) See Appendix

creases in muscle mitochondria. The significance of these changes in relation to the problem of acclimation to cold will be discussed later after the results of the "in vitro" measurements of AA incorporation and the determination of free leucine concentration in plasma and various tissues of the CA rat have been presented. The reason is that it is difficult to give a concrete interpretation of any measured difference of "in vivo" rates of labelled AA into mitochondrial proteins in terms of mitochondrial protein synthesis without having estimated or controlled the pool of the direct precursors, in this case free plasma leucine and free tissue leucine.

3. Mitochondrial protein synthesis in isolated mitochondria from liver, BAT and skeletal muscle of WA, CA and CE rats.

a) Purpose of the experiment: The main purpose was to measure specifically mitochondrial protein synthesis (which the "turnover experiment" did not measure at all, and the "in vivo" experiments measured only partially) with less interference from the extramitochondrial protein synthesizing system in order to determine if the observed changes during the development of cold-acclimation and the differences between the WA and CA rats could (or could not) be attributed to the mitochondrial protein syntesizing system of BAT, liver and skeletal muscle . It is known that the rate of AA incorporation in isolated mitochondria can be related to various physiological states of several tissues, as was discussed in the introduction and shown in Table 2.

b) Principle of the technique: The rate of ^{14}C-leucine incorporation in isolated mitochondria was measured in an appropriate medium in the presence of an ATP-regenerating system, cycloheximide, a bicine buffer pH 7.4 and under conditions which minimize bacterial contamination (Wheeldon and Lehninger 1966; Beattie 1967, a and 1970).

- The use of an ATP-regenerating system: Two fundamentally different media with respect to the source of energy necessary for the formation of the peptide bonds are in general used for the measure of AA incorporation activity in isolated mitochondria (Wheeldon and Lehninger 1966): the respiration supported incorporation medium and the "ATP-regenerating system" supported medium. The above-mentioned authors showed, in a comparative study of the two systems, that the hypothetical high-energy intermediates of oxidative phosphorylation generated by respiration of electron transport are not obligatory energy sources for mitochondrial protein synthesis as it can be supported at maximal rates by an ATP-regenerating system in the presence of the uncoupler oligomycin. These authors demonstrated also that this system is far more effective in supporting AA incorporation than the respiration supported system. Thus, it seems that the whole complex enzymatic apparatus of respiration can be excluded as a required component of mitochondrial protein synthesis. This is a useful property for the studies of AA incorporation capacity in mitochondria isolated from CA rats, which are often obtained in

an uncoupled state if necessary precautions are not taken. As uncoupled mitochondria have little or no capacity for synthesizing ATP, the supply of ATP could be rate limiting in AA incorporation studies, with isolated mitochondria from CA rats. In order to avoid this, and to work under optimal conditions, we decided to use an ATP-regenerating system.

- <u>The use of cycloheximide</u>: Although the mitochondrial preparations were washed systematically four times in order to minimize microsomal contamination, cycloheximide was added in order to inhibit any possible microsomal incorporation (Beattie 1967, a).

- <u>The use of a bicine buffer</u>: It was shown by Hamberger et al. (1969) that the use of bicine buffer should be preferred to various other buffers which are inhibitory especially at high concentrations.

- <u>Bacterial contamination</u>: All reasonable precautions were taken (cf. "Material and Methods") to minimize bacterial contamination during the isolation and incubation of mitochondria. It was discussed before that it is not possible to avoid completely bacterial contamination but that relatively high concentrations of bacteria have to be added to mitochondria prepared under sterile conditions in order to observe a significant bacterial contribution to the total incorporation. Moreover, a nondependence on ATP could be expected if bacteria were solely responsible for the observed incorporation.

c) Description of the experiment: 32 rats were divided in 8 experimental groups with four rats in each group. The groups and the average weight of the rats were:

GROUP	SPECIFICATION	WEIGHT (in grams)
1. Young WA rats	0 days in the cold	237 ± 1.4
2. Young CE rats	4 days in the cold	196 ± 9.8
3. Young CE rats	14 days in the cold	245 ± 32.1
4. Young WA rats	WA control of group 3	277 ± 14.5
5. CA rats	6-8 weeks in the cold	307 ± 12.3
6. WA rats	WA control of group 5	371 ± 14.9
7. CA rats	6-8 weeks in the cold and then 1 day in the warm immediately before the experiment	308 ± 7.9
8. WA rats	6-8 weeks in the warm and then 1 day in the cold immediately before the experiment	352 ± 6.9

Two or four rats of corresponding control and experimental groups were used simultaneously in each experimental day. Liver, BAT and skeletal muscle mitochondria were isolated and incubated in different media containing various ATP concentrations and processes as described under "Material and Methods". The various specific activities for the different experimental groups plotted against ATP concentrations are shown in figure 19 (liver), figure 20 (BAT), and figure 21 (muscle).

d) Results:

Liver: Figure 19 shows that no significant difference was observed at any ATP concentration between mitochondria isolated from the four different experimental groups: WW (warm-acclimated rats in the warm), CC (cold-acclimated rats in the cold), WC (warm-acclimated rats - 1 day in the cold), CW (cold-acclimated rats - 1 days in the warm). The activity was markedly dependent on ATP concentration, high concentrations of ATP have an inhibitory effect. Maximum incorporation occurred at 1 mM ATP.

Brown adipose tissue: Figure 20 shows that after 4 days of cold-exposure, an inhibition of incorporation occurred at all ATP concentrations; the capacity for AA incorporation increased markedly after 2 weeks of exposure and decreased thereafter to levels slightly higher than those of the unacclimated rat. There was also an increase of leucine incorporation, mainly at high ATP concentrations when the adult WA rat was left the day before the experiment in the cold and a slight decrease of incorporation at all ATP concentrations when the CA rats was left the day before the experiment in the warm. The incorporation was strongly dependent on ATP concentration, the maximum activity being observed at a concentration of 2 mM ATP.

Skeletal muscle: Figure 21 shows that there is a decrease of AA incorporation 4 days after the exposure of the young WA rat to cold which remain at those levels during the following 10 days. Thereafter, there is an increase in AA incorporation in muscle mitochondria from the CA rats which results in activities equal or slightly higher (mainly at high ATP concentrations) than the

Figure 19: Specific activities of isolated liver mitochondria, incubated at various concentrations of ATP, under the conditions described under "Material and Methods" of WA rats in the warm (WW), CA rats in the cold (CC), WA rats exposed to cold one day (WC), CA rats exposed to warm one day (CW). The vertical lines represent the standard errors of the mean.

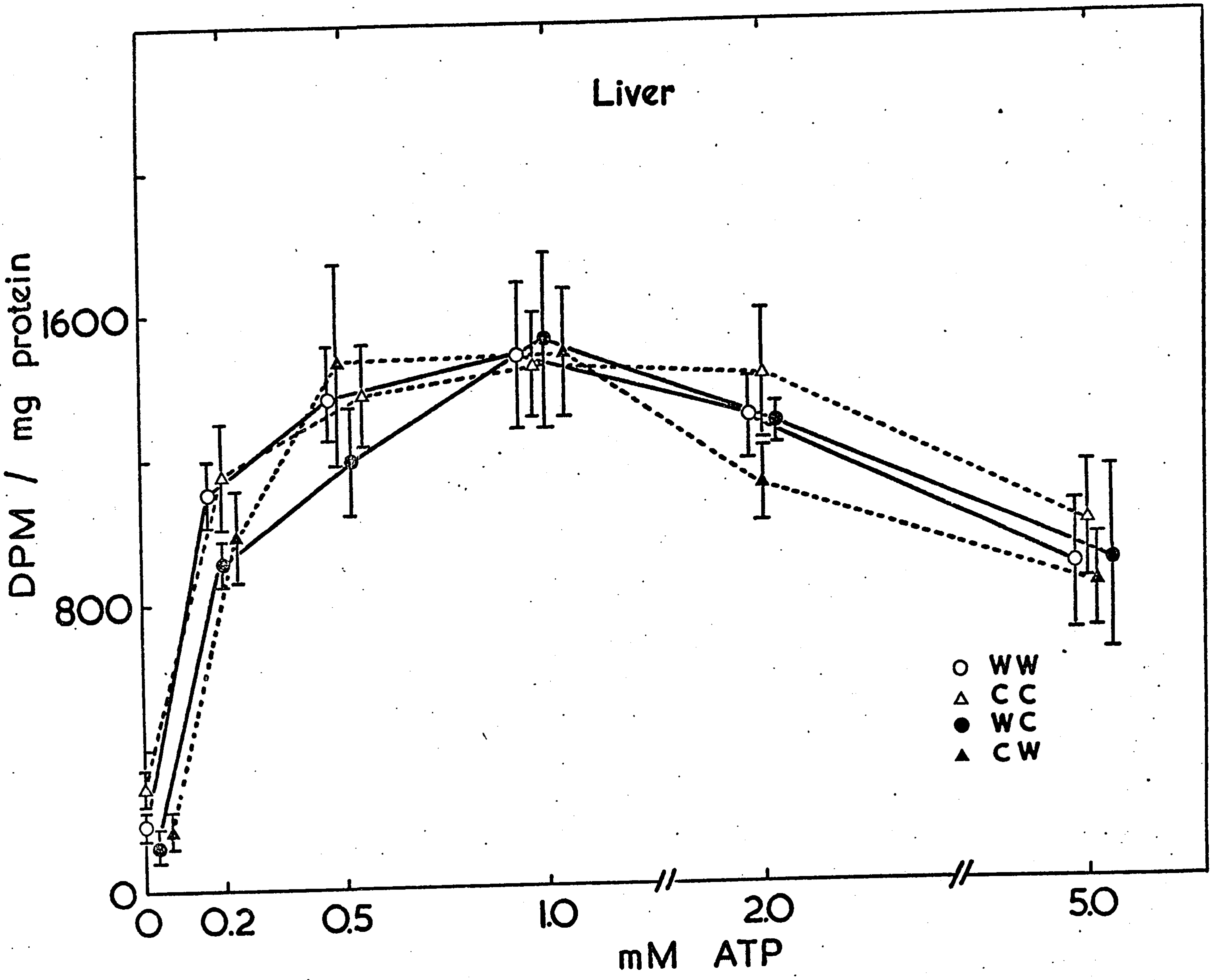

Figure 19

Figures 20 and 21: Specific activities of isolated BAT mitochondria (Fig. 20) and skeletal muscle mitochondria (Fig. 21) incubated at various concentrations of ATP under the conditions described under "Material and Methods" at various times of cold-exposure or cold-acclimation.

Blue bars represent specific activities of WA rats
White " " " " of CA "
Striped " " " " of WA "exposed 1 day to cold
Dotted " " " " of CA " " 1 day to warm
The vertical lines represent the standard errors of the mean.

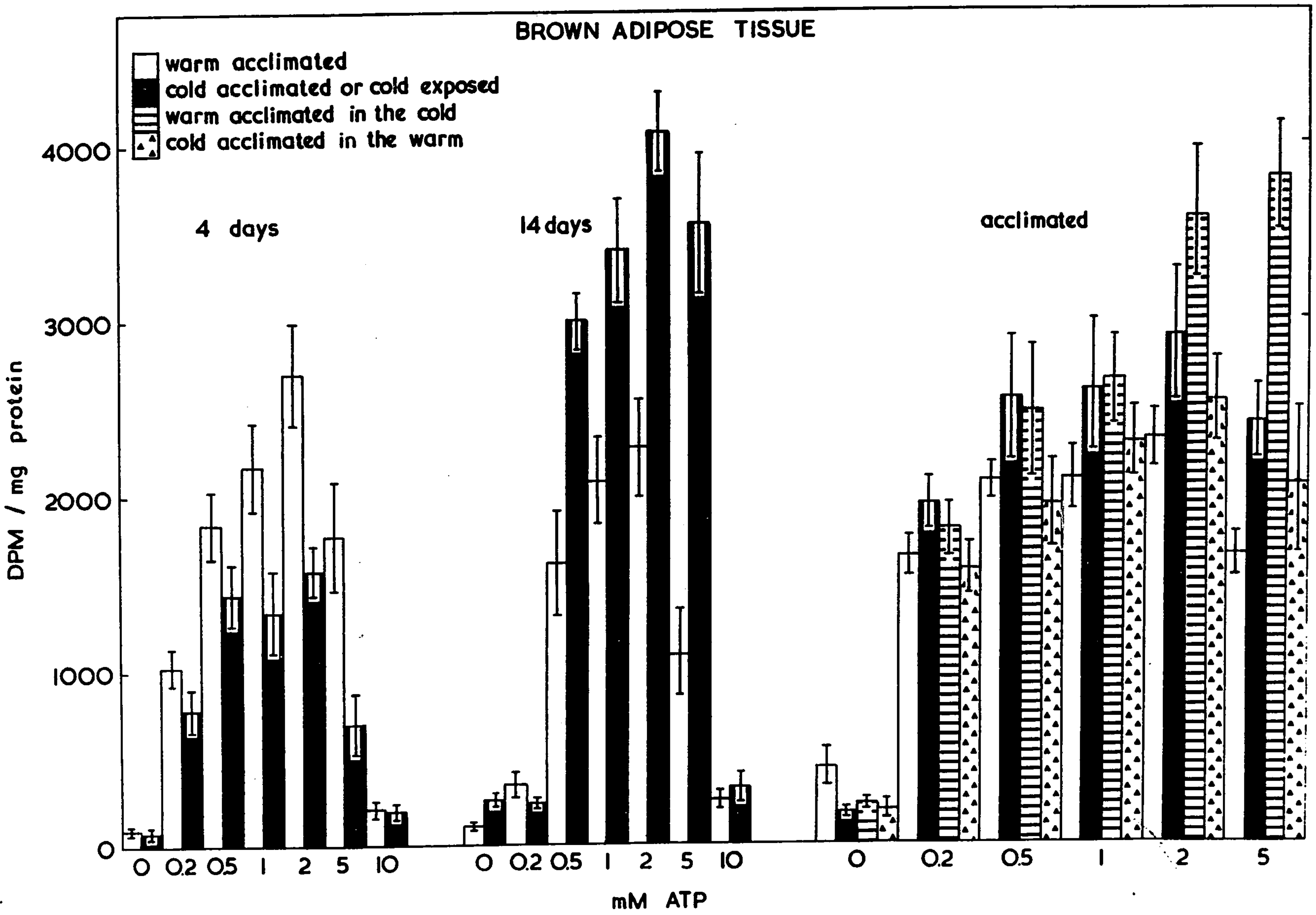

Figure 20

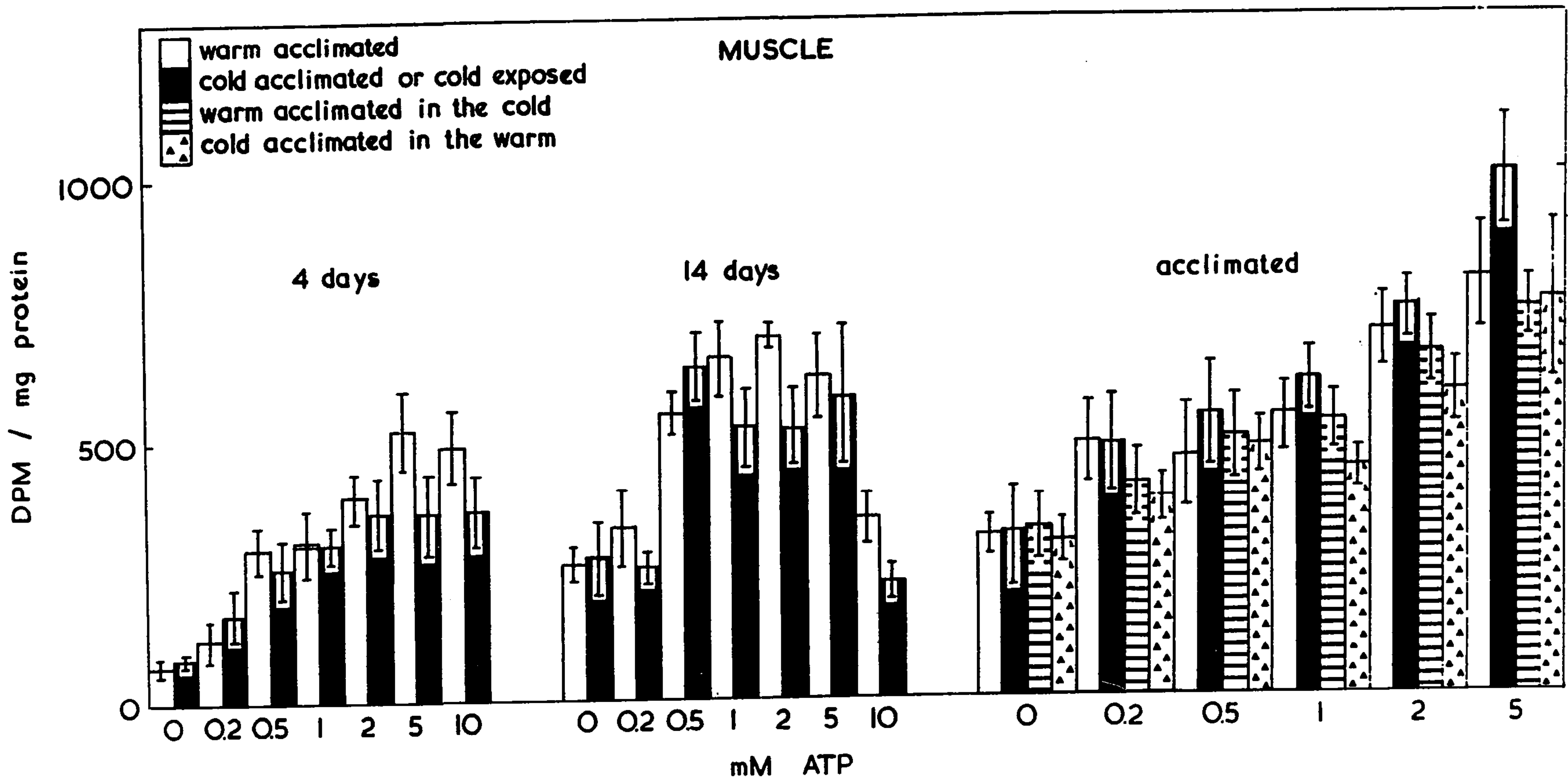

Figure 21

activities of the corresponding WA group. The maximum of incorporation in the various experimental groups occurs at 5 mM ATP. Slight differences in the capacity of AA incorporation were also observed when the WA rat was exposed to cold for 1 day or when the CA rat was exposed to warm for 1 day.

A comparison of the maximal specific activities between figures 19, 20 and 21 shows that BAT mitochondria have a much higher capacity for incorporating AA than liver or skeletal muscle mitochondria.

e) Discussion: The comparison between figures 20 and 21 shows that the marked increase in AA incorporation of BAT mitochondria during the first 2 weeks of acclimation to cold is accompanied by a decrease in the rate of AA incorporation in muscle mitochondria. By contrast, during the last period of the adaptation to cold (from the second week to the 6-8th week) the contrary is observed: AA incorporation in BAT mitochondria decreases, whereas AA incorporation in muscle mitochondria increases. This suggests that major changes in mitochondrial protein synthesis take place relatively quickly in BAT mitochondria during the first two weeks of acclimation to cold, but that changes in muscle mitochondrial protein synthesis are slower, which is in accordance with the slow turnover of the muscle organelle. That the observed changes in BAT and muscle mitochondria are related to adaptation to cold is suggested by the fact that they take place specifically in tissues which are important sites of NST (BAT and skeletal muscle) and not in liver mitochondria isolated from CA rats, WA rats, CA rats exposed one day to warm immediately be-

fore the experiment, WA rats exposed one day to cold immediately before the experiment (See Fig. 19). Moreover, it seems unlikely that these changes are directly related to heat production by NST because NST is switched off in isolated mitochondria, but they are probably related to the capacity for making NST which can be considered as a permanent manifestation of a tissue, at least during a certain time (cf. Introduction).

It should be noted that it is not known in which specific mitochondrial proteins the AA incorporation takes place, but it is generally believed that isolated mitochondria do incorporate AA in relatively insoluble mitochondrial fractions which are normally associated with the "contractile" protein, the "structural" protein or the "other cytochromes" fractions and not in water-soluble proteins (cf. Introduction).

A comparison of the maximal specific activities obtained for BAT, liver and skeletal muscle mitochondria (See figures 19, 20 and 21) shows that under identical conditions of incubation, BAT mitochondria have a much higher capacity for AA incorporation than liver or skeletal muscle mitochondria, especially in rats which have lived two weeks in the cold. Moreover, in the three studied tissues, the incorporation was strongly dependent on ATP concentration. Wheeldon and Lehninger (1966) have observed a similar ATP effect. This suggests that ATP concentrations "in vivo" could be an important factor in the regulation of mitochondrial protein synthesis.

As was discussed in the introduction and shown in Table 2, the capacity for AA incorporation was related to the physiological state of several different tissues. However, few of these studies have compared the "in vitro" with the "in vivo" rates of AA incorporation. Only recently, Greenawalt et al. (1972) have measured both rates during the morphogenetic development of Neurospora crassa. The authors have shown that during the germination period, the ability of Neurospora mitochondria to respire and catalyze coupled phosphorylation increases dramatically and was correlated with "in vitro" as well as with "in vivo" increases of AA incorporation. As the mitochondrial energy coupling is dependent on the molecular architecture of the mitochondrial membranes, the studies made with Neurospora crassa suggest that there exists a direct correlation between mitochondrial protein synthesis and the functional state of the organelle. Figure 22 shows a comparison of the "in vivo" and the "in vitro" rates of AA incorporation in BAT mitochondria during acclimation to cold with the experiments made by Greenawalt with Neurospora crassa (1972). The pattern of the variation of the respiratory coefficient of BAT mitochondria as measured by Smith and Roberts (1967) is also included. It should be noted that the BAT mitochondria as isolated by these authors are probably uncoupled to an unknown degree as no ADP/o ratios were measured.

Considered that during acclimation to cold there is a marked hypertrophy and hyperplasia of BAT (Smith and Horwitz 1969) which is accompanied by a marked increase in the number of mitochondria

Figure 22: A comparison of BAT changes in mitochondrial QO_2, mitochondrial "in vivo" and "in vitro" AA incorporation during acclimation to cold with corresponding variations observed during the development of Neurospora crassa cells. BAT mitochondrial QO_2 values were taken from Smith and Roberts (1967) and the Neurospora crassa values from Greenawalt et al. (1972).

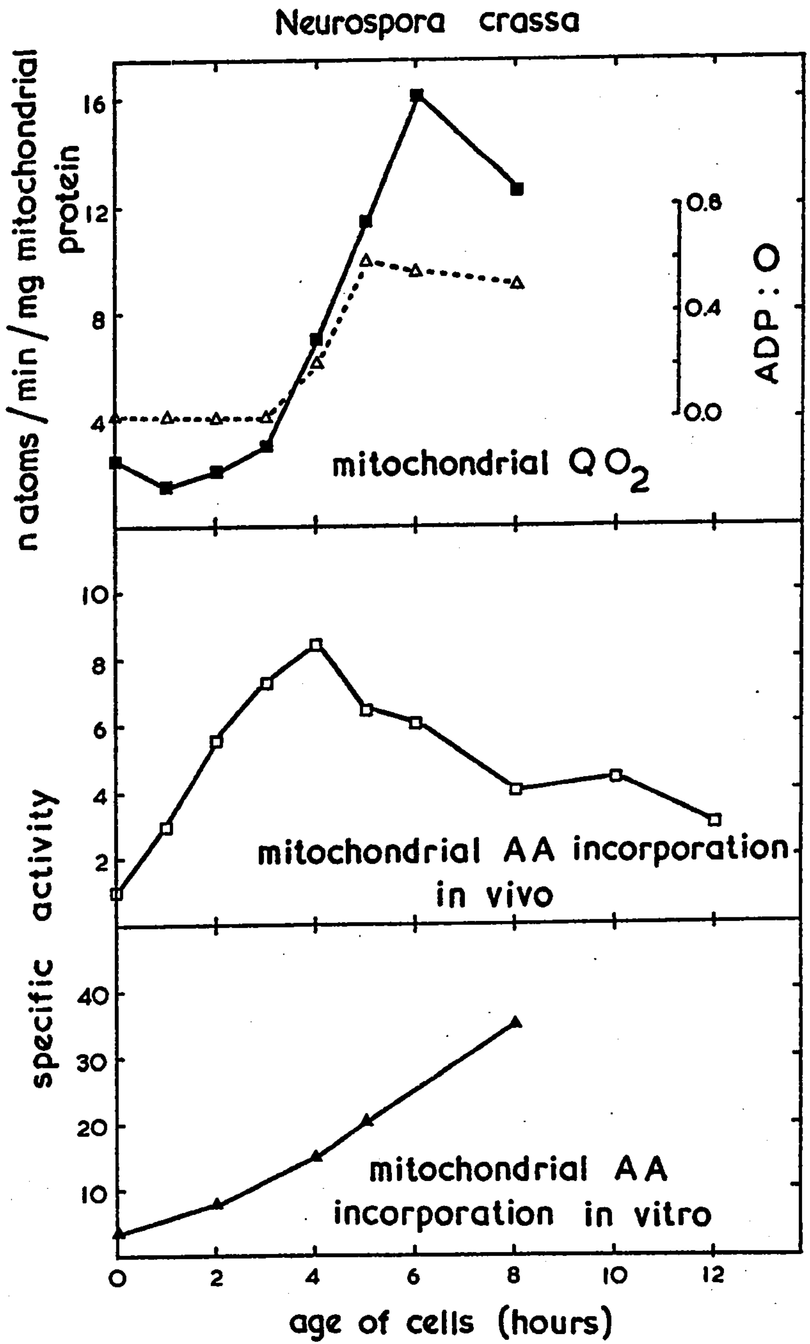

Figure 22

per cell, by an increase in the number of cristae per mitochondrion (Suter 1969; Thomson et al. 1969; Skala et al. 1970), by an increase in the total cytochromes per unit of mitochondrial protein (Skala et al. 1970; Barnard et al. 1970; Jansky et al. 1969) and by an increase in the specific activity of cytochrome oxidase (Skala et al. 1970), it is postulated that as in the case of Neurospora crassa there is in BAT a direct correlation between mitochondrial protein synthesis and the functional state of that organelle.

It is also possible that this correlation is not only limited to BAT, but extends to certain other tissues of the rat and that the increased mitochondrial protein synthesis in BAT mitochondria which occurs during the first two weeks of exposure to cold is directly or indirectly responsible, not only for the development of BAT, but also for the development of the CA state. This hypothesis is based on the oxytetracycline experiments which were discussed in the introduction and where it was shown that inhibition of protein synthesis in BAT mitochondria by oxytetracycline was paralleled by an inhibition of the development of the enhanced response to NA.

4. Determination of leucine concentration in various tissues of the WA, CA and cold-exposed rat.

a) Purpose of the experiment: Previous experiments have shown that on several occasions there was an apparent inhibition of leucine incorporation in various tissues of the CE and CA rats.

However, at least two different types of evidence have suggested that this inhibition of AA incorporation could not systematically be interpreted in terms of inhibition of mitochondrial protein synthesis. As a matter of fact: (*)

- in turnover experiments, the regression lines of the various mitochondrial fractions of the different tissues were systematically drawn at lower levels of specific activity than in the WA rats (See figures 11 a to 11 u). This was noticed even with the liver or kidney mitochondria where no change in the turnover values between WA and CA rats were measured. If there was a real inhibition of 20-30% of mitochondrial protein synthesis and no change in the mitochondrial turnover, there should be almost no mitochondria in these tissues after a period of 2-3 weeks in CA rats.

- a comparison of the rates of AA incorporation in liver mitochondria of CA rats measured by the "in vitro" and "in vivo" techniques (See figures 19 and 16) shows that there was no inhibition of AA incorporation in isolated mitochondria from CA rats, whereas an inhibition of AA incorporation of about 20-30% was noticed in the livers of CA rats as measured by "in vivo" techniques.

This suggested that other factors than an inhibition of mitochondrial protein synthesis could be responsible for the measured

(*) See Appendix

inhibition of AA incorporation. An inhibition of AA incorporation could theoretically be caused by:

- a real inhibition of mitochondrial protein synthesis
- an increase in the concentration of free leucine in plasma and/or in various tissues, which would result in a dilution of the labelled injected leucine
- an inhibition of some step of leucine transport to the various sites of mitochondrial protein synthesis*
- by a preferential utilization of the injected leucine for other purposes than for mitochondrial protein synthesis
- by a combination of several of these possible effects.

In order to have at least some information about the pools of leucine, it was decided to estimate total free leucine concentration in plasma, liver, BAT and skeletal muscle of CA rats.

b) <u>Principle of the technique</u>: Leucine concentration was determined on the AA Autoanalyzer as described under "Material and Methods".

c) <u>Description of the experiment</u>: 25 rats were divided in 5 experimental groups:

*(Portugal et al, 1970)

GROUP	NUMBER OF RATS	WEIGHT (in grams)
1. Young WA rats	4	201 ± 4.9
2. Young WA rats exposed to cold 3 days	4	172 ± 1.4
3. Young WA rats exposed to cold 14 days	5	202 ± 5.1
4. WA rats	6	412 ± 17.4
5. CA rats	6	342 ± 21.1

The plasma, BAT, liver and skeletal muscle were processed as described under "Material and Methods". The leucine concentration expressed in micromoles/100 ml of plasma or micromoles/100 g of wet tissue is reported in figure 23.

d) <u>Results</u>:

<u>Plasma</u>: after 3 days of cold-exposure of the WA animal there was a 60% increase of leucine concentration in plasma which decreased thereafter in the CA rat, but remained at slightly higher levels than in the WA rat.

<u>BAT</u>: there was a continuous increase in leucine concentration during acclimation to cold.

<u>Liver</u>: after a slight initial increase, no change was observed during acclimation to cold.

<u>Muscle</u>: no change was observed during acclimation to cold.

Figure 23: Leucine concentrations in plasma, liver, IBAT and skeletal muscle of WA, CA and cold-exposed rats. The numbers under the bars indicate 1 : 0 days in the cold, 2 : 3 days in the cold, 5 : 14 days in the cold, 3 : WA (6-8 weeks in the warm), 4 : CA (6-8 weeks in the cold). The open bars represent WA rats and the black bars cold-exposed or CA rats. The vertical lines represent the standard errors of the mean.

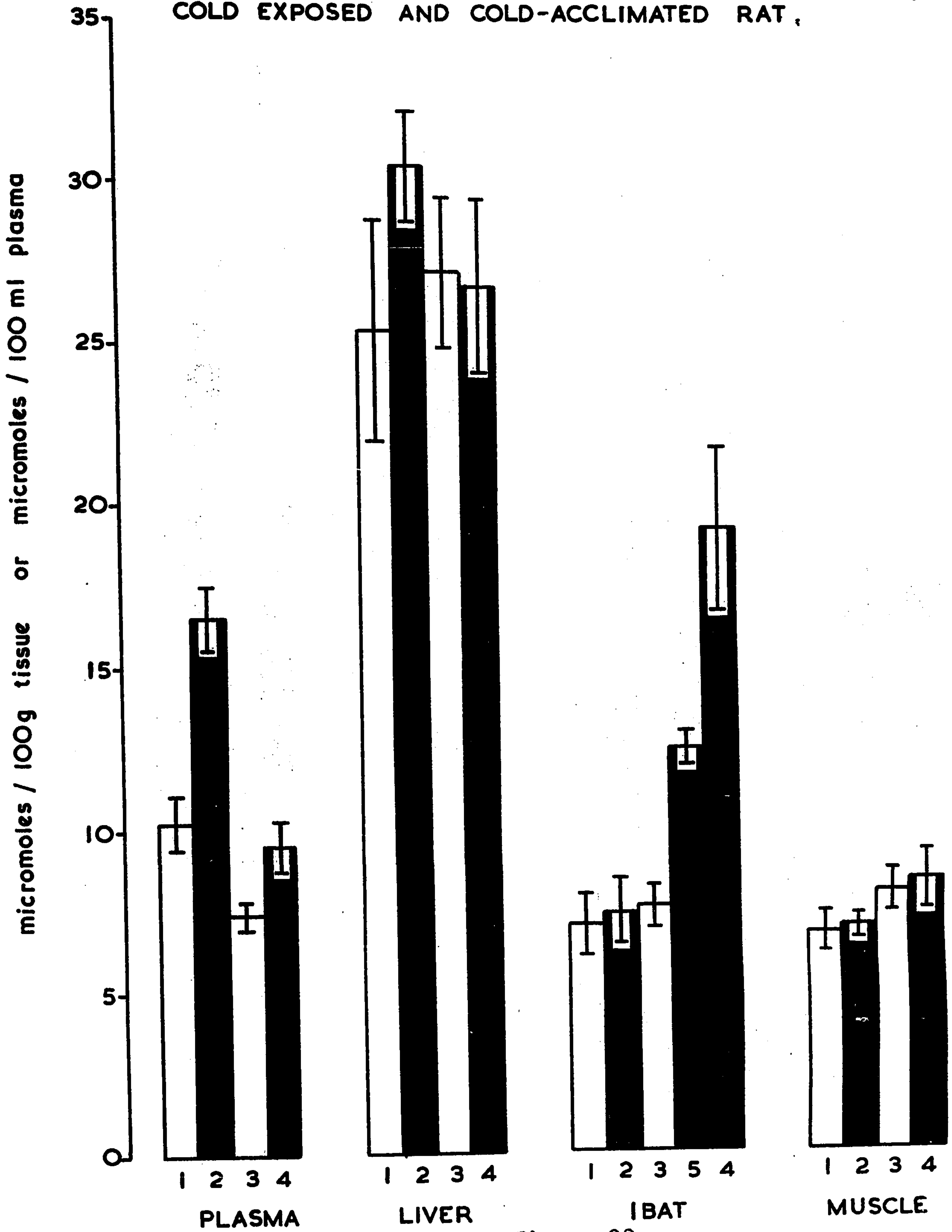

Figure 23

e) Discussion: Leucine concentration changes were observed during acclimation to cold mainly in plasma and BAT and not in muscle or liver tissue. Such an increase in plasma leucine was observed by Williams et al. (1950) after the cold-exposure of WA animals. This increase was not specific for only leucine but was observed with many other AA and is possibly related to changes in protein metabolism such as the active gluconeogenesis which takes place in the rats just after their cold-exposure and which decreasesduring cold-acclimation (Penner and Himms-Hagen 1968).

An increase of about 60% of the plasma leucine concentration 3 days after the cold-exposure of the young WA rat is expected to affect proportionally mitochondrial protein synthesis in various tissues. In fact, there is a systematic inhibition of the "in vivo" AA incorporation in various tissues of the WA rat exposed to cold (See figures 13, 14 and 15). However, it seems that the marked increase in leucine concentration is only a partial explanation for the unspecific inhibition of "in vivo" AA incorporation after cold-exposure, mainly for two reasons: First, the 60% increase in plasma leucine concentration is not large enough to account for the marked inhibition of leucine incorporation in muscle mitochondria of 3 days cold-exposed rats (Fig. 15), and cannot also explain the different effects observed in liver (Fig. 13) and BAT (Fig. 14) mitochondria. (*)

Moreover, the marked increase in leucine concentration of BAT during acclimation to cold is probably related to the growth and development of the tissue during that period. It could result from an increased leucine uptake from plasma or from a decreased

(*) See Appendix

leucine utilization. This increase in BAT leucine concentration could affect the results of the "in vivo" measure of AA incorporation in BAT only by multiplying the observed increase of incorporation that takes place in BAT after the exposure of the rat to cold by a certain unknown factor, which accounts for the dilution of the label. Such a multiplication by a factor could theoretically be done only when more information could be obtained about compartmentation problems.

In summary, this study of leucine concentration in various tissues of the rat has drawn the attention to one important problem which is often ignored, that is the influence of the pool of precursors on the "in vivo" measures of the rates of protein, DNA, RNA phospholipids and other compounds, under physiological condition where changes are expected to happen, not only in the rate of macromolecule synthesis, but also in the pools of their precursors.

V. GENERAL DISCUSSION AND CONCLUSION

The principal aim of the experiments described in this thesis was to answer the question: "Is the mitochondrial protein synthesizing system directly involved in and responsible for the development of NST during acclimation to cold of the rat?"

The following experimental approaches were used:

- the determination of the half-life of mitochondrial proteins and of various mitochondrial protein fractions in the intact animal
- the measure of the "in vivo" ^{14}C-leucine incorporation in mitochondrial protein and various mitochondrial protein fractions together with the determination of free leucine concentration in plasma and various tissues
- the determination of the "in vitro" AA incorporation in mitochondrial proteins.

Four tissues were studied: IBAT and skeletal muscle as they are both important sites of NST; liver and kidney as they are not considered important sites of NST.

It was shown that there is a decrease in the half-life of BAT and skeletal muscle mitochondria of CA rats which is associated mainly with relatively insoluble mitochondrial protein fractions.

It was discussed in the introduction that some proteins of these fractions are believed to be made specifically by the mitochondrial protein synthesizing system. No decrease in half-life was observed in the soluble mitochondrial protein fractions of these tissues nor in any of the different mitochondrial protein fractions of liver and kidney. This provides evidence for:

- there is an alteration in metabolism of certain proteins synthesized by mitochondria in CA rats
- this alteration is associated with NST, i.e. it occurs in those tissues in which NST takes place and not in those tissues in which NST does not take place.

As all the mitochondrial protein fractions obtained by the procedure described under "Material and Methods" represent groups of proteins and not individual proteins with an estimated pool size, the actual rate of synthesis cannot be calculated from the data presented. Differences in estimated turnover values of specific proteins can only be interpreted in terms of changes of synthesis when the pool size of these specific proteins is known. (*) Therefore, the observed decrease in the measured half-life of insoluble BAT and skeletal muscle mitochondrial protein fractions may be due to a change in the rate of synthesis and/or degradation of one or more proteins in the group of proteins or to a change in the amount of one or more proteins in the group, or it may be associated with both changes. Thus, the measured de-

(*) See Appendix

crease in the half-life of BAT and skeletal muscle mitochondria suggested that mitochondria of these tissues may be different in CA rats as compared to WA rats.

In order to have more information about this problem and also about the problem of the origin of the proteins synthesized by mitochondria in CA rats, it was decided to study AA incorporation in various mitochondrial protein fractions by a combination of "in vivo" and "in vitro" techniques, not only after acclimation to cold, but also during the development of the CA state.

Two different experimental approaches were used because, although the "in vivo" technique possesses all the qualities of the direct "in situ" experimental approaches, it does not measure specifically AA incorporation into proteins made by the mitochondrion as the "in vitro" technique does. However, as was discussed under "Results", if rats are killed after a short period of time following the intravenous injection of ^{14}C-leucine, the protein synthesized specifically by the mitochondrial protein synthesizing system are more likely to be labelled first. That this assumption is basically correct is suggested by the fact that during acclimation to cold the variations observed in the patterns of AA incorporation measured by both "in vivo" and "in vitro" techniques are similar and also by the fact that "in vivo" insoluble proteins incorporate more AA than the soluble ones (with the exception of skeletal muscle mitochondria).

From the results obtained by these different experimental approaches, the following interpretation is made: (See Table 5)

- after cold-exposure of the young WA rat, there is an inhibition of AA incorporation in BAT and skeletal muscle mitochondria and possibly also in liver mitochondria. This inhibition was observed in BAT and skeletal muscle mitochondria by both "in vitro" and "in vivo" techniques. The observed "in vivo" inhibition of incorporation is probably accentuated, but cannot be explained, by the increase in plasma leucine concentration which is probably diluting the injected labelled leucine. It was discussed that other factors than increased plasma leucine concentration could affect the AA incorporation in various tissues of cold-stressed rats.

- during acclimation to cold, the patterns of AA incorporation in liver, BAT and skeletal muscle mitochondria differ markedly: in BAT mitochondria there is an increase in AA incorporation at 14 days which decreases thereafter during the last period of cold-acclimation, but which remains at higher levels in the CA rat than in the WA rat; in muscle mitochondria, the contrary is observed, the initial decrease of AA incorporation is maintained during two weeks and the incorporation increases only during the last period of the adaptation to levels equal to or slightly higher than of the WA rat; in liver mitochondria no major difference is observed during all the period necessary to acclimate a rat to cold.

T A B L E 5

Summary of changes in "in vivo" and "in vitro" amino acid incorporation into mitochondrial proteins of Brown adipose tissue, skeletal muscle and liver during cold-acclimation. (For explanation of symbols see footnote to table)*.

BROWN ADIPOSE TISSUE

	IN VIVO			IN VITRO
Days in cold	Structural protein fraction	Contractile protein fraction	Total proteins	Total proteins (at 2mM ATP)
12 h	<	=	<	
3 days	=	> ?	< ?	
4 days				<
14 days	>	>	=	>
6-8 weeks	=	=	=	> ?

SKELETAL MUSCLE

	IN VIVO			IN VITRO
Days in cold	Structural protein fraction	Contractile protein fraction	Total proteins	Total proteins (at 5mM ATP)
12 h	<	<	<	
3 days	<	<	<	
4 days				< ?
14 days	<	<	<	=
6-8 weeks	< ?	<	<	> ?

Table 5 continued

LIVER

	IN VIVO			IN VITRO
Days in cold	Structural protein fraction	Contractile protein fraction	Total proteins	Total proteins (at 1 mM ATP)
12 h	<	<	<	
3 days	< ?	=	<	
14 days	<	< ?	<	
6-8 weeks	<	< ?	<	=

* < significantly less in cold than in warm

< ? slightly less in cold than in warm

= equal in cold and warm

> ? slightly greater in cold than in warm

> significantly greater in cold than in warm

The principal conclusion of this thesis and the answer to the question which was asked in the introduction is that changes in the mitochondrial protein synthesizing system of BAT and skeletal muscle mitochondria, but not of liver mitochondria are associated with the development of NST during acclimation to cold of the rat. That these changes are directly or indirectly responsible for the development of NST during acclimation to cold and for the maintainance of the CA state, is suggested by the fact that the two major types of variation i.e. the decrease in mitochondrial half-lives as well as the changes in AA incorporation were both specifically observed in tissues which are important sites of NST (skeletal muscle and BAT) or which have a major regulatory function in the development of NST (BAT) and not in tissues which are not important sites of NST (kidney and liver).

Many biochemical and morphological changes have been observed in BAT mitochondria during acclimation to cold, but in comparison little information is available for muscle mitochondria. It is known that inhibition of mitochondrial protein synthesis with oxytetracycline during the early stages of cold-acclimation prevents the development of NST (Himms-Hagen 1971 and 1972, b). Two weeks after the exposure of the rats to cold, the inhibition of the enhanced calorigenic response to NA was associated with the inhibition of the normal increase in cytochrome oxidase specific activity in BAT which accompanies the marked hypertrophy and hyperplasia of that tissue and which is associated with increases in the number of mitochondria per cell, in the number of cristae per mitochondrion and in the total cytochromes per unit of mitochondrial protein. However, no inhibition in skeletal

muscle mitochondria occurred until 3-4 weeks. This sequence in mitochondrial changes agrees with the sequence of the observed increases of AA incorporation in BAT and skeletal muscle mitochondria. In fact, during the first two weeks of cold-acclimation, there was a marked increase in BAT mitochondrial AA incorporation which was followed by an increase in skeletal muscle mitochondrial AA incorporation during the last period of the adaptation. It is possible that the changes might be susceptible to inhibition by oxytetracycline and be required for the development of cold-acclimation. Oxytetracycline might have inhibited the synthesis of a few mitochondrial proteins which were required specifically for the development of acclimation to cold.

This hypothesis implies not only that mitochondria are relatively dynamic organelles but also that the mitochondrial synthesizing system has a fundamental role in the regulation of energy metabolism. In fact, as discussed in the introduction, it seems that the dynamic model of Ashwell and Work (1970) , essentially based on the diversity of half-lives of the various mitochondrial components, represents the best picture of the actual knowledge of mitochondrial biogenesis ,and studies made with the Neurospora crassa and yeast have demonstrated that there exists a direct correlation between mitochondrial protein synthesis and the functional state of the organelle in these microorganisms.

It is postulated from the results presented in this thesis and from several other discussed lines of evidence that:

- changes in the capacity for producing increased quantities of heat by NST are accompanied by changes in the metabolism of certain proteins synthesized by the mitochondria of BAT and skeletal muscle which result in changes in the structure and function of BAT and skeletal muscle mitochondria;

- as in yeast or Neurospora crassa mitochondria (cf. Introduction), there is in BAT and skeletal muscle mitochondria a direct correlation between mitochondrial protein synthesis and the functional state of the organelles during and after acclimation to cold;

- the increased BAT mitochondrial protein synthesis which occurs during the first two weeks of cold-exposure of the rat is responsible, not only for the development of that tissue, but also directly or indirectly for the development of the CA state.

Further work should be done in this direction but it seems that the basic problem which has to be solved, not only for the understanding of acclimation to cold, but for the general understanding of the regulation of energy metabolism, is the determination of the nature and function of the proteins made by the mitochondrial protein synthesizing system.

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VII. APPENDIX (*)

DISCUSSION OF THE PROBLEMS OF MEASURING PROTEIN SYNTHESIS AND TURNOVER IN VIVO

The turnover of a substance (Jeffay, 1963) may be defined as the rate of synthesis (or entry) or the rate of degradation (or removal) of the substance. In a non-growing healthy animal, (in "steady state") the rates would be equal. In general, three different experimental approaches have been used for the measurements of turnover rates (Jeffay, 1963; Poole, 1971).

Method A: The single injection of a labeled precursor:

The turnover rate can be calculated from the equation:

$$\frac{dSb}{dt} = \frac{V}{B}(Sa - Sb)$$

where Sa = specific activity of the precursor A of the product B

Sb = specific activity of the product B

V = velocity of the reaction

B = amount of product B

V/B = turnover rate.

Method B: The maintenance of the labeled precursor at constant specific activity:

The equation which applies to this method is:

$$\frac{Sb}{Sa} = 1 - e^{V/B}$$

(*) References at the end of the appendix.

and the turnover time is given by the measurement of the exact time when the specific activity of the product (Sb) will be equal to the specific activity of the precursor (Sa).

<u>Method C</u>: The measure of the rate of disappearance of the label from previously labeled proteins:

This method is based on two assumptions. First, substantially all the incorporation of the labeled precursor into the product should occur before the first specific activity measurement is performed. Second, that the differential equation describing the decay of the specific activity from the product has the form

$$\frac{dSb}{dt} = -\frac{V}{B}(Sa - Sb)$$

Each of these three methods presents major practical problems. Not only does the amount of product have to be known but also the specific activity of the immediate precursor and of the product. For method B the specific activity of the immediate precursor must be kept constant throughout the experiment. For method C there must be no reutilization of labeled material produced by degradation of the product. In the case of the mitochondrial protein fractions studied in the experiments described in this thesis B represents a mixture of several proteins or enzymes, many of which are not yet identified, and as a consequence neither B nor Sb can be known. Moreover, Sa cannot be estimated either because mitochondrial proteins originate from several different compartments of unknown precursor pool size (Portugal et al., 1970; Mortimore et al., 1972; Hider et al., 1969 and 1971). When Sa, Sb or B are not precisely known, the term "half-life" should be preferred to the term turnover. In certain cases it is sometimes possible to

minimize some of the problems of the determination of the half-life of mitochondrial proteins. In fact, it was shown recently by several authors (Swick et al., 1968; Druyan et al., 1969; Aschenbrenner et al., 1970) that the reutilization of the labeled AA precursor which influences the determination of turnover or half-life rates can be reduced by the use of nonreutilisable precursors such as guanidine-labeled arginine or δ-aminolevulinic acid. However, the practical application of these two precursors is limited: the former is only useful for liver cells (as the Krebs-Henseleit cycle is specifically confined in them) and the latter for the determination of the turnover of haem-containing proteins. Because of their limited application, these two precursors were not useful for the achievement of the principal goal of this thesis, which was to determine in a systematic study whether there was an alteration in the metabolism of different groups of mitochondrial proteins in various tissues of WA and CA rats. It was therefore decided to study the problem of the biosynthesis and degradation of mitochondrial groups of proteins by a combination of three different experimental approaches because each of them possessed <u>different</u> and <u>complementary</u> advantages and disadvantages, which could possibly help us in the interpretation of our results.

The three different methods used as well as their respective interpretation problems were:

<u>Method 1</u>: the determination of the half-life of mitochondrial proteins and of various mitochondrial protein fractions in various tissues of the intact animal, by the measure of the rate of disappearance of ^{14}C-leucine from previously labeled proteins. An observed difference of the half-life of a given mitochondrial protein fraction between WA and CA rats suggests that this mitochondrial fraction may be different,

but does not provide any information about the nature of this difference because it could result from a change in the rate of synthesis and/or degradation of one or more proteins in the group of proteins or to a change in the amount of one or more proteins in the group or it may be associated with both changes.

Method 2: the measure of the "in vivo" ^{14}C-leucine incorporation in various mitochondrial protein fractions together with the determination of free leucine concentration in plasma and various tissues.

This method has the advantage of being a direct "in vivo" approach for the estimation of mitochondrial protein synthesis. However, the interpretation of the results of AA incorporation is complicated by two main factors: a) Permeability factors: the AA before reaching the different sites of mitochondrial protein synthesis has to cross several membranes such as the plasma membrane, the endoplasmic reticulum membrane, the inner mitochondrial membrane, etc. For instance, Wheeldon and Lehninger (1966) observed that there was a rapid uptake of AA into the liver intramitochondrial pool which was energy-linked and possibly due to a specific transport mechanism. Moreover, Hider et al., (1971) working with isolated skeletal muscle, suggested that AA were incorporated directly in proteins from the extracellular pool. It must be remembered that mitochondrial proteins are made at two different intracellular sites from two different pools of AA. The measurement of their radioactivity was made at a short time (5 minutes) after the injection of labeled material in order to assess principally the activity of the mitochondrial system (Beattie, 1966). b) Dilution factors: the injected AA will be diluted in plasma, extracellular fluid,

cytoplasmic and mitochondrial pools, the size of which is for the most part unknown (Hider et al., 1969; Mortimore et al., 1972). In this context Portugal et al., (1970) have shown that the amount of lysine in liver mitochondria and other cell organelles is considerably smaller than the amount in the cytoplasm. Moreover, a change in the turnover of leucine in any one of the different pools would also alter the extent of the dilution.

The results of the "in vivo" experiments presented in this thesis were interpreted in function of the free leucine concentration in plasma and of the total leucine concentration in the various tissues at the moment of the injection of the AA. (For a discussion see pp. 140-144).

Method 3: the determination of the "in vitro" AA incorporation in mitochondrial proteins.

This technique has the advantage over the preceding ones of measuring incorporation into proteins made specifically by mitochondria. Moreover in this method several membrane permeability problems or factors related to the possible dilutions of the label in plasma or cytoplasm do not have to be considered. However, in isolated mitochondria many regulating factors of mitochondrial protein in the intact animal are likely to be absent or altered (Hawley and Greenawalt, 1970). However, it was shown previously (p. 45 and Table 2, p. 49) that the "in vivo" AA incorporation can be related to the physiological state of the tissue in many instances. Analysis of Table 5 which compares the AA incorporation studies made by the "in vivo" and "in vitro" approaches as well as the comparison of figures 16,17,18 to 19,20,21 shows that in general there is a good agreement between the pattern of AA incorporatio[n] observed by both techniques during acclimation to cold.

In summary, it is clear that none of these three methods is exempt from criticism, but the interpretation of our results (See chapt. V) is based on the combined information obtained from three different experimental approaches which have complementary qualities and defects.

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