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RAT BROWN ADIPOSE TISSUE MITOCHONDRIA AND NONSHIVERING THERMOGENESIS

by Michel Desautels

A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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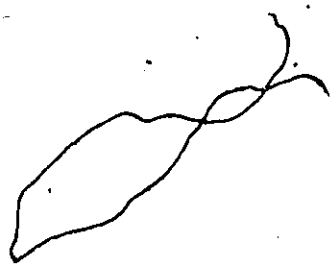

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ABBREVIATIONS

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
BAT	brown adipose tissue
BSA	bovine serum albumin
B.W.	body weight
C-A	cold-acclimated
CHX	cycloheximide
CmH^+	proton conductance
CoA	coenzyme A
cyclic AMP (cAMP)	adenosine-3', 5'-monophosphate
cyclic GMP (cGMP)	guanosine-3', 5'-monophosphate
DNP	dinitrophenol
ΔE	membrane potential
Δp	proton electrochemical gradient
ΔpH	transmembrane pH gradient
E	epinephrine (adrenaline)
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide, oxidized form
FCCP	carbonylcyanide-p-trifluormethoxy-phenylhydrazon
FFA	free fatty acids
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid
IBAT	interscapular brown adipose tissue
mCCP	carbonylcyanide-m-chlorophenylhydrazon

M.W.	molecular weight
NAD	nicotinamide adenine dinucleotide, oxidized form
NE	norepinephrine (noradrenaline)
NST	nonshivering thermogenesis
OTC	oxytetracycline
SAL	saline
SDS	sodium dodecyl sulfate
SE	standard error
SHE	Sucrose, Hepes, EDTA; isolation media for mitochondria
T_4	thyroxine
TES	N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid
W-A	warm-acclimated
WAT	white adipose tissue

SUMMARY

During acclimation of the rat to cold brown adipose tissue grows and its mitochondria proliferate. Brown adipose tissue is known to be the major site in the cold-acclimated rat of nonshivering thermogenesis, the process by which extra heat is produced and body temperature maintained when the rat is in a cold environment. The extra heat produced is known to be controlled by the sympathetic nervous system and cold-acclimated rats have an enhanced capacity to respond to catecholamines by an increase in metabolic rate (calorigenic response). Brown adipose tissue mitochondria are known to be especially adapted for heat production. They possess a unique and controllable proton conductance pathway, sensitive to purine nucleotides, that allows a high rate of respiration (and hence of heat production) when open. The mechanism underlying the very large capacity of brown adipose tissue for heat production involves futile proton cycling across the mitochondrial inner membrane, dissipating as heat the energy normally utilized in other types of mitochondria for ATP production.

The work described in this thesis has as its objective the description of some of the changes in mitochondrial composition, structure and function which occur in brown adipose tissue during acclimation of the rat to cold and the elucidation of the mechanisms by which such changes are brought about.

Results show that acclimation to cold is best described as occurring in two phases. The first phase is termed acute cold stress, involves rapid changes and occurs during the first few hours of exposure to cold. The second phase, termed the adaptive phase, involves slower changes and extends from the first day to approximately the second week

of exposure to cold.

The first phase, termed acute cold stress, is characterized by an increase in purine nucleotide binding to brown fat mitochondria (a measure of the amount of proton conductance pathway), which is not accompanied by any change in the proportion of the 32 000 M.W. polypeptide, known to be the binding site of the purine nucleotides on the mitochondrial inner membrane. Mitochondria are larger and have an altered internal structure resulting in an increased inner membrane surface. A similar increase in purine nucleotide binding can be produced in vivo by an intravenous infusion of norepinephrine and in vitro by incubating isolated mitochondria in KCl medium at alkaline pH. Mitochondrial internal structure is also altered following these treatments. The norepinephrine-induced increase in purine nucleotide binding is totally reversible and protein synthesis is not required for either the norepinephrine or the cold-induced increase in purine nucleotide binding. Thus, the initial response to cold stress appears to involve unmasking of mitochondrial proton conductance pathways, most probably mediated by norepinephrine. The actual mechanism of unmasking is still unknown but may involve a reorganization of mitochondrial membranes.

During the adaptive phase, which occurs during prolonged cold exposure, there is a large increase in brown adipose tissue mass and a proliferation of mitochondria within the tissue. Mitochondria are also larger and their cristae more numerous and arranged in a parallel fashion. Simultaneously, there is also an increase in the proportion of the 32 000 M.W. polypeptide accompanied by an additional increase in purine nucleotide binding resulting in a higher concentration of proton conductance pathways. All these changes regress rapidly and at the same

rate when cold-acclimated rats are returned to a warm environment. The increase in purine nucleotide binding sites is dependent on cytosolic but not mitochondrial protein synthesis. Although the tissue hypertrophy may itself be mediated by norepinephrine, the changes in mitochondrial composition are not.

Thus, an increase in the mitochondrial concentration of proton conductance pathways, which occurs simultaneously with tissue hypertrophy and mitochondrial proliferation constitutes the adaptive response to cold and most probably contributes to the enhanced capacity of the rats for nonshivering thermogenesis. However, the in vitro mitochondrial respiratory capacity does not appear to be directly dependent on the concentration of proton conductance pathways and a regulatory function for the increase in proton conductance sites in mitochondria of cold-acclimated rats is suggested.

Several questions remain unanswered, particularly concerning the mechanism of action of norepinephrine, the identification of the hormone(s) responsible for the development of brown adipose tissue and of the changes in its mitochondria as well as the physiological importance of the unmasking observed in the acute phase of cold exposure. Further research is needed to explain more fully in biochemical terms the calorigenic action of norepinephrine on brown adipose tissue and the nature of the adaptive changes in brown adipose tissue mitochondria which underlie the increased capacity of cold-acclimated rats to respond calorigenically to norepinephrine.

INTRODUCTION

Over the last few years, brown adipose tissue has been the subject of intensive research to explain its role and function in living organisms. Its importance in the process of hibernation has long been recognized. This tissue was originally called the "hibernating gland" although its actual role was poorly understood. However, the dominant role of this small tissue, representing only few per cent of total body weight, in heat production has only been firmly established very recently. New research trends also point to an important role in overall energy balance not only in small mammals and newborns, but also possibly in adult humans.

Brown adipose tissue is basically an energy dissipating organ, a biological furnace. Its specialization in heat production is reflected by its composition and function. When the metabolism of brown adipose tissue is stimulated by cold exposure or at the time of arousal from hibernation, the adipocyte oxygen consumption and heat production increase. Norepinephrine is known to mediate this response. Brown adipose tissue has indeed a large capacity for fatty acid oxidation. About half the volume of a brown fat cell is occupied by triglyceride droplets (fuel) and the other half by mitochondria (furnace). Brown adipose tissue mitochondria are also specially adapted for a high oxidative metabolism. They possess an unusually high content of substrate dehydrogenases and oxidative enzymes. In addition, mitochondrial respiration can escape normal respiratory control so that a high rate of respiration (heat production) may be sustained from substrate (fatty acids) oxidation without stoichiometric production of ATP. A special mechanism underlying this uncoupling of oxidative phosphorylation has

recently been described (proton conductance pathway) and will be discussed thoroughly in the literature review.

The capacity of animals for nonshivering heat production is related to the presence of brown adipose tissue which is found in larger amounts in cold-acclimated animals, hibernators and neonates of various species including man.

Two different responses of brown adipose tissue to cold exposure may be distinguished. There is a rapid response characterized by an increase in oxygen consumption and heat production upon acute cold stress. This reaction may be easily reproduced in vitro by addition of norepinephrine to a suspension of isolated brown adipocytes. This response is present in brown fat cells of all animals independently of species or adaptation differences. However, the thermogenic capacity of brown adipose tissue from hibernators and cold-acclimated animals is much greater than that of brown adipose tissue from non-hibernators and non-acclimated animals. Several laboratories have been carrying out research to elucidate the mechanism of the calorigenic action of norepinephrine with a particular attention to the mechanism of induction of mitochondrial thermogenesis.

A slower response of brown adipose tissue to cold exposure of non-hibernators involves the enhancement of its capacity for heat production. The tissue grows and long-term changes occur in the mitochondrial composition and function. Few data are available concerning this adaptive phase of cold acclimation and the interrelationships between the acute and adaptive phases have not been clearly defined. The mechanism underlying mitochondrial energy dissipation described in hibernators may probably be extrapolated to mitochondria of brown adipose

tissue of cold-acclimated or newborn animals, known to have a maximum and comparable thermogenic capacity. However, its development and importance at various times during adaptation of non-hibernators to cold have not been studied intensively. Similarly, hormone(s) responsible for the development and transformation of brown adipose tissue and of its mitochondria during adaptation to cold is (are) not fully identified.

The work described in this thesis concerns the changes in mitochondrial energy dissipation in brown adipose tissue of cold-acclimated rats, in which the tissue is enlarged and has an enhanced capacity for thermogenesis. The composition, structure and function of mitochondria isolated from brown adipose tissue of rats exposed to cold for short and long time periods is described. Changes which develop in the energy-dissipating mechanism during exposure to cold are studied and an attempt is made to identify the hormone(s) responsible for their development.

Finally, the mechanism of the cold-induced changes is studied, with particular reference to the role of protein synthesis and to the nature of the intracellular mediator.

A detailed background for the work described in this thesis is given in the literature review that follows. The literature review is divided into several sections corresponding to the different subject areas touched upon in the study. A summary of present knowledge of cold adaptation is followed by a detailed discussion of possible mechanisms of intracellular heat production. Then, the role and function of brown adipose tissue is reviewed with a special emphasis placed upon its mitochondria. Mitochondrial composition and structure as well as the mechanism of energy dissipation unique to this tissue is reviewed. This

is followed by a description of the chain of events occurring during adaptation to cold and by a discussion of the control of thermogenic activity in the acute and adaptive phases.

LITERATURE REVIEW

PART I. COLD-ACCLIMATION AND NONSHIVERING THERMOGENESIS

A. Nature and definitions

Mammals are described as warm-blooded animals because of their ability to maintain a blood temperature higher than that of their environment. They are characterized by a remarkable capacity to regulate their body temperature so that it remains constant under a great variety of environmental conditions. The maintenance of body temperature is a delicate balance between heat production and heat loss. Heat is produced by metabolic processes. Metabolism is a term which refers to the various chemical reactions characteristic of the living cell and heat is a by-product of these reactions. The more active the metabolism, the greater is the heat production. When a rat is exposed to a cold environment (that is below its thermoneutral temperature, experimentally, 4-6°C), its heat loss is minimized by piloerection and vasoconstriction. This reduction of heat loss is under sympathetic control (124). The extra heat needed to maintain its body temperature constant is produced mainly in muscle by shivering. The rat thus transferred from a warm to a cold environment will be referred as being "cold-exposed". After having been in the cold for a fairly long period (3-4 weeks), the rat is able to maintain its body temperature constant without shivering (124). It is said to be "cold-acclimated" and to rely on nonshivering thermogenesis (NST) for its heat production. Nonshivering thermogenesis is then the production of heat by acceleration of metabolic processes other than those involved in shivering thermogenesis. It must

however be pointed out that either shivering or nonshivering thermogenesis are "regulatory heat production" mechanisms not to be confused with the essential and obligate heat production resulting from energy exchange and metabolism necessary for the life of every cell (92). As such, they do not include the basal metabolic activity. The "regulatory heat production" is a facultative thermogenesis which may or may not be activated according to the environmental conditions. According to Leblanc (174), what appears to be of importance in adaptation to cold is the suppression of shivering which is non-economical (142) and mentally disturbing. In fact, the relative contribution of shivering and nonshivering thermogenesis upon cold exposure varies with the adaptive state of the animal or depends upon some specific built-in capacity of the animal to use NST. For instance, newborns and hibernators such as the hamster, the hedgehog or the ground squirrel possess an inherent capacity to use NST although newborns lose it relatively early in life while hibernators keep it through their adult life. In certain non-hibernators such as the rat, guinea pig, mouse and rabbit, nonshivering thermogenesis appears to be an adaptive process being only present in the adult to any appreciable extent after adaptation to cold. In man or in some other large mammals such as the sheep, the capacity to use NST is lost early after birth and the adult appears to be unable to undergo to any appreciable extent the necessary adaptation for non-shivering thermogenesis (125).

There is considerable evidence that nonshivering thermogenesis is under the control of the sympathetic nervous system and that the capacity to use nonshivering thermogenesis, whether adaptive (as in the cold-acclimated rat) or constitutive (as in the hamster, an hibernator) is

associated with a capacity to respond to catecholamines by a large increase in metabolic rate (calorigenic response) which may be as great as 4-5 x the basal metabolic rate (124). This enhanced calorigenic response in the cold-acclimated rats is best observed during an intravenous infusion of catecholamines (Fig. 1). The level of NE (norepinephrine) in the plasma during parallel infusions is actually lower in the cold-acclimated rats than in the warm-acclimated animals and compares quite well with the concentration needed to stimulate oxygen uptake by tissues in vitro (60). It has been suggested that the plasma level of NE during the infusion approximates the synaptic concentration of NE during cold exposure. Thus, the enhanced calorigenic response to NE in cold-acclimated rats is not due to higher circulating levels of NE as suggested by Leblanc and Pouliot (175).

Since mammals are aerobic organisms and heat is a by-product of metabolic activity, the rate of heat production is equal to the rate of respiration. In the rat (the animal under study in this thesis) as in most species, the enhancement of the calorigenic response to catecholamines develops slowly during the first four weeks of cold exposure. During this period, the extent to which the rat shivers while in the cold decreases slowly and reaches a minimum at about the same time as the calorigenic response to catecholamines reaches a maximum (124). (Compare Fig. 2 and 3).

Nonshivering thermogenesis can now thus be defined as:

an INCREASE IN HEAT PRODUCTION not due to muscle movements, above (an not including) the basal metabolic activity

which is FACULTATIVE, i.e. being subject to an on/off switch under the control of the sympathetic nervous system

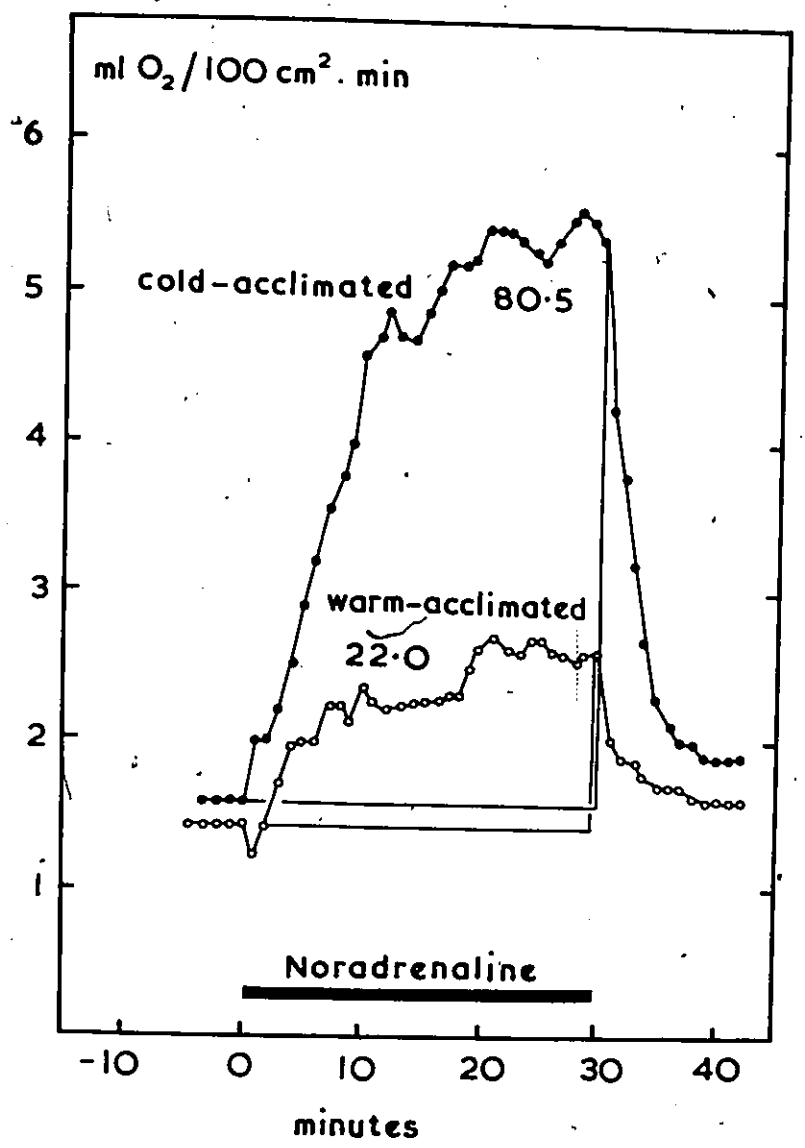

FIG. 1. Enhancement of calorigenic response to norepinephrine (noradrenaline) by cold acclimation. Oxygen uptake of a warm- and a cold-acclimated rat during infusion of norepinephrine is shown.

Norepinephrine was infused intravenously (0.5µg/100 cm^2 per min.) from 0 to 30 min. Rats were lightly anesthetized with sodium pentobarbital. Warm-acclimated rat weighed 478 g, and cold-acclimated rat weighed 373 g; they had lived at room temperature (25-28°C) and in the cold (4°C) respectively, for 13 weeks, and their weights at the start of the acclimation period were 202 g and 192 g respectively. Values of 80.5 and 22.0 on the graph are obtained from the area under the curve during the 30 min. of infusion of norepinephrine, and they represent total increase in oxygen uptake in millimeters of O_2 per 100 cm^2 in 30 min. Increases shown are typical of rats kept under these conditions. (From Himms-Hagen (124)).

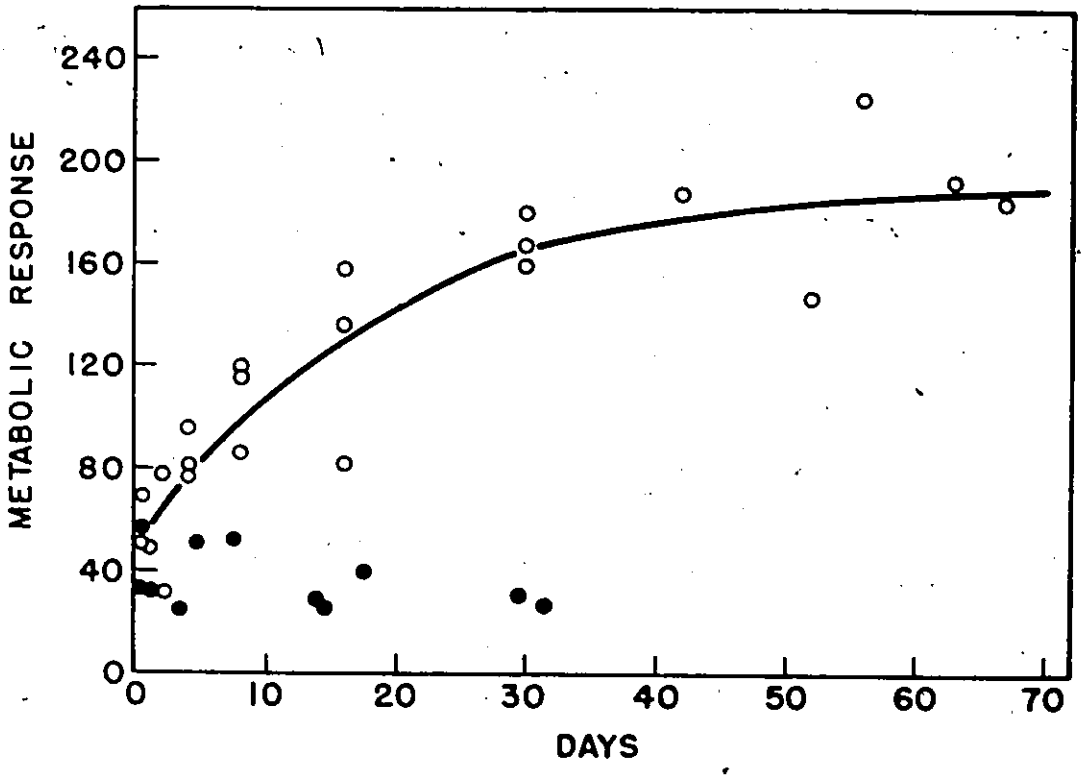

FIG. 2. Development of enhanced calorigenic response to noradrenaline during acclimation to cold.

Metabolic response to intravenously infused noradrenaline at level of 1 µg free base per minute per rat in rats previously maintained at 30°C (●) and 6°C (o) is shown. Metabolic response is given in square centimeters and corresponds to the area under the curve of oxygen consumption vs. time during noradrenaline infusion (100 min) minus area corresponding to initial oxygen consumption in same period of time. The average increase in ml O_2 consumed per minute for each rat, during infusion of noradrenaline, can be obtained by dividing metabolic response units by 20.
(From Depocas (57)).

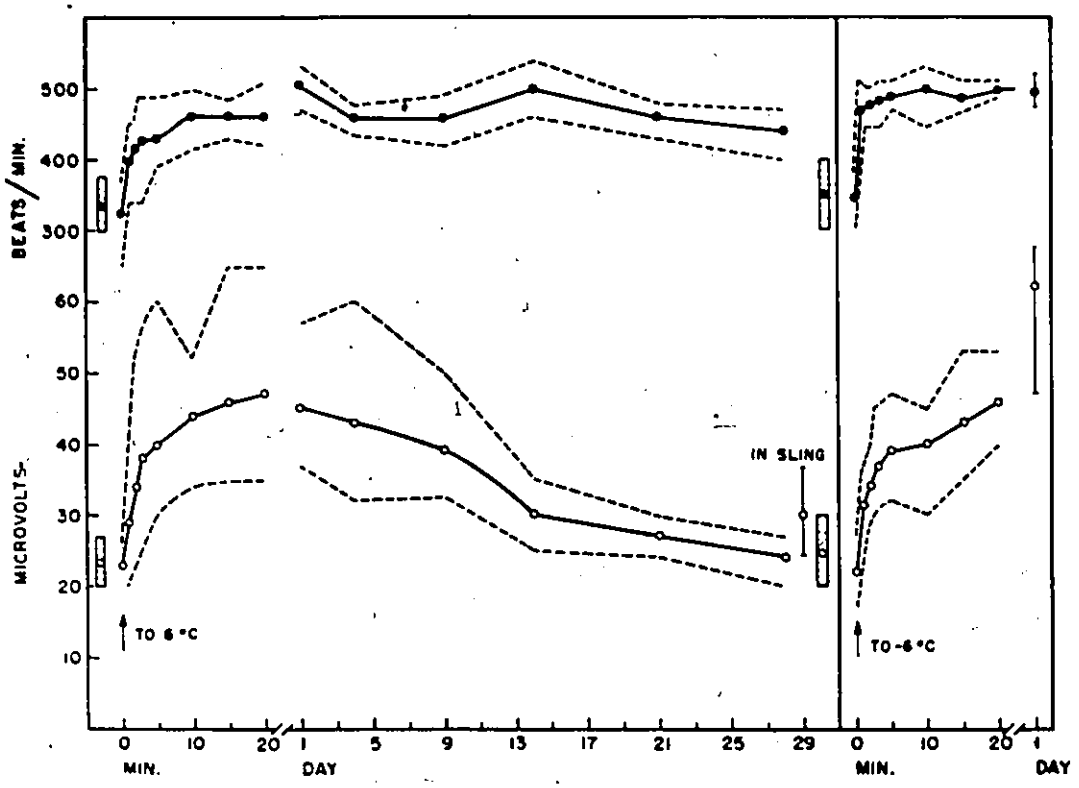

FIG. 3. Increase and decrease in shivering as measured by electrical activity, during acclimation to cold.

Muscle electrical activity as related to time of exposure to 6°C is shown (solid line) in the left-hand part of the diagram (lower tracing). Broken lines show range of variation. Vertical bars at 0 and 29 days show muscle electrical activity in warm-acclimated rats living at 30°C. Upper tracing shows heart rate. At 30 days cold-acclimated rats were moved to -6°C (right-hand part of the diagram).
(From Hart et al., (110).

and ADAPTIVE, i.e. in animals with no built-in capacity for use of NST the process is present in the adult to any appreciable extent only after adaptation to living in the cold.

The ultimate goal of the research on NST is the full explanation in biochemical terms of the large increase (4X) in metabolic rate upon catecholamine administration in animals adapted to use NST. Any explanation or hypothesis to be accepted must satisfy the main characteristics of NST as defined above. In studying the factors that regulate NST, it will be necessary to distinguish between those that are associated with the rapidly-occurring responses initiated when the animal is acutely exposed to cold from those that are associated with the changes induced by prolonged cold exposure. In this regard, a cold environment refers to any temperature below the thermoneutral one of the animal and sufficient to induce some adaptive changes. However, in this thesis, the term "cold-acclimated" will refer to animals who have lived at 4-6^{o}C for a minimum of 3-4 weeks and "cold-exposed" will refer to animals exposed to 4-6^{o}C for a shorter period.

B. Sites of nonshivering thermogenesis

The major site of NST appears to be Brown Adipose Tissue (BAT) (84, 85). Although it is not possible to measure directly from which organs NST originates, an indication of the regional importance in heat production can be obtained by measuring blood flow to various organs in the intact animal. Earlier work estimated the contribution of BAT to NST from cold-acclimated rats not to exceed about 6-10% of the total heat production (126, 127, 155). The contribution of skeletal muscle was estimated at about 50% (155, 156). However, recent work by Foster pointed out that the contribution of BAT had been considerably under-

estimated (84, 85). Measurements of blood flow with microspheres pointed to BAT as the dominant site of the calorigenesis induced by norepinephrine, accounting for 60% of the calorigenic response of the cold-acclimated rats. Skeletal muscle could not be responsible for more than 12% of the calorigenic response to NE in CA rats. More recent work performed on conscious warm- and cold-acclimated rats also demonstrated the dominant role of brown adipose tissue in the replacement of shivering by nonshivering thermogenesis as can be seen in Fig. 4 (86).

The contribution of BAT to the increased thermogenesis of WA rats exposed to cold (4°C) is fairly large but is clearly much more dominant in CA animals. Muscle shivering is important in WA animals but becomes minor in CA animals exposed to low temperature.

Further evidence for the important role of BAT in NST is the correlation between the amount of brown fat and the metabolic response of the animal to NE (39). The existence of a large calorigenic response to NE is always associated with the presence of abundant deposits of BAT (39, 264). Brown adipose tissue also undergoes dramatic changes in structure, composition and metabolic characteristics when the capacity of the animal to use NST changes (39, 264). The changes taking place in BAT upon cold acclimation will be described in detail in a later chapter.

Skeletal muscle, even though it is a minor contributor to NST in small mammals, also undergoes changes during cold adaptation (14). For comparison, a short review of the changes occurring in skeletal muscle will also be presented in a later chapter. It is also important to note that the relative contribution of BAT and skeletal muscle to NST in small mammals such as the rat might be different from the contribution in large cold-acclimated animals, such as the seal for example, where

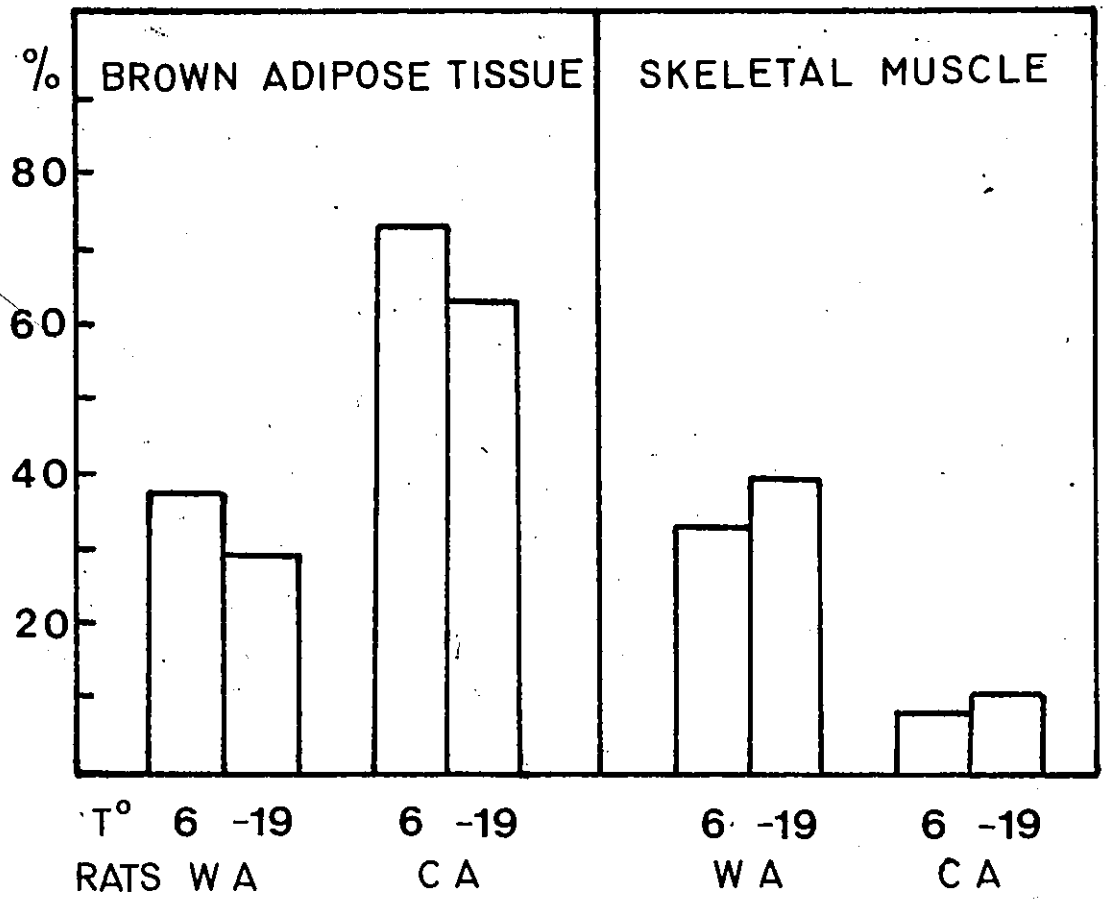

FIG. 4. Approximate contribution of tissues to cold-induced increase in metabolic rate in warm- and cold-acclimated animals.

The changes in blood flow to various tissues of cold-exposed (6°C or -19°C) W-A or C-A rats is expressed as percentages of the sum of the increases in flow to the tissues. The changes in flow were calculated by taking flow at 25°C (W-A rats) or 21°C (C-A rats) as the base.
(From Foster and Frydman (86)).

the relative contribution of skeletal muscle may be of greater importance.

PART II. BIOCHEMICAL MECHANISMS OF HEAT PRODUCTION

From a thermochemical point of view, a human or an animal may be regarded as just a catalyst for the combustion of foodstuffs. Indeed, it has also been long recognized that the production of heat is associated with respiration and the concomitant oxidation of foodstuffs. The amount of energy released during combustion of a substrate is essentially the same whether it occurs in a test tube or in a living cell. However, in a cell, the process is stepwise and controlled. The purpose of this combustion is usually not the production of heat but rather the production of a useful form of conserved energy, namely, ATP. From calculations based on the free energy of combustion of various substrates and intermediates, it appears that virtually all the energy released during oxidation occurs in the reaction of reduced mitochondrial substrates (such as NADH or succinate) with O_2 (221, 222). The mitochondria are presently accepted as being the major site of heat production in a mammalian cell. However, some (if not all) of the energy released during mitochondrial oxidation is conserved in the form of ATP. There is still controversy about the relative proportion of the energy conserved as ATP or dissipated as heat (i.e. about the efficiency of coupling of the oxidative phosphorylation) (126). As regard to heat production; the main questions are then how much energy is conserved or dissipated upon mitochondrial oxidation and how the process is regulated and activated.

There are several theories about the way cells can make ATP or preserve energy but the prevailing one at present is the "chemiosmotic" hypothesis. The theory was developed between 1961 and 1966 by Mitchell who postulated the existence of a gradient of electrochemical potential

of hydrogen ions across membranes competent in respiratory phosphorylation (186, 187). Basically, the oxidation of substrates drives protons across the mitochondrial inner membrane normally impermeable to protons (Fig. 5, site a); the energy-rich compound ATP is formed when the protons flow back through a complex of enzymes, the ATP synthetase (Fig. 5, site c). Actually, the proton gradient established by oxidative electron transport (Fig. 5, site a) represents a store of free energy. It is, as well as ATP, a convertible form of energy in the living cell that can be utilized for a whole variety of types of work (259).

The factors controlling the rate of mitochondrial respiration vary according to the state of coupling of the mitochondria. In the tightly coupled state (where electrons cannot pass through the electron transport chain without generation of ATP), the rate of respiration is regulated mainly by the phosphorylation state ratio ATP/ADP-P_i (73, 209). The mitochondrial adenine nucleotide translocator maintains a high ATP/ADP ratio in the cytosol relative to the intramitochondrial space and has been demonstrated to be the rate-limiting enzyme for oxidative-phosphorylation in vitro (119, 171) and in vivo (1). The electron transfer reactions are apparently always near equilibrium with the phosphorylation reaction within the mitochondria and deviations from equilibrium by small changes in the ratio of internal ATP and ADP have large effects on the rate of electron transfer. Thus, as long as the oxidation is tightly coupled to phosphorylation, the rate of fuel combustion will be controlled by the requirement for ATP. It is then obvious that one way of obtaining an increase in heat production would be to increase the activity of ATP hydrolysing systems. It must be pointed out that in such cases, the heat produced would result mainly

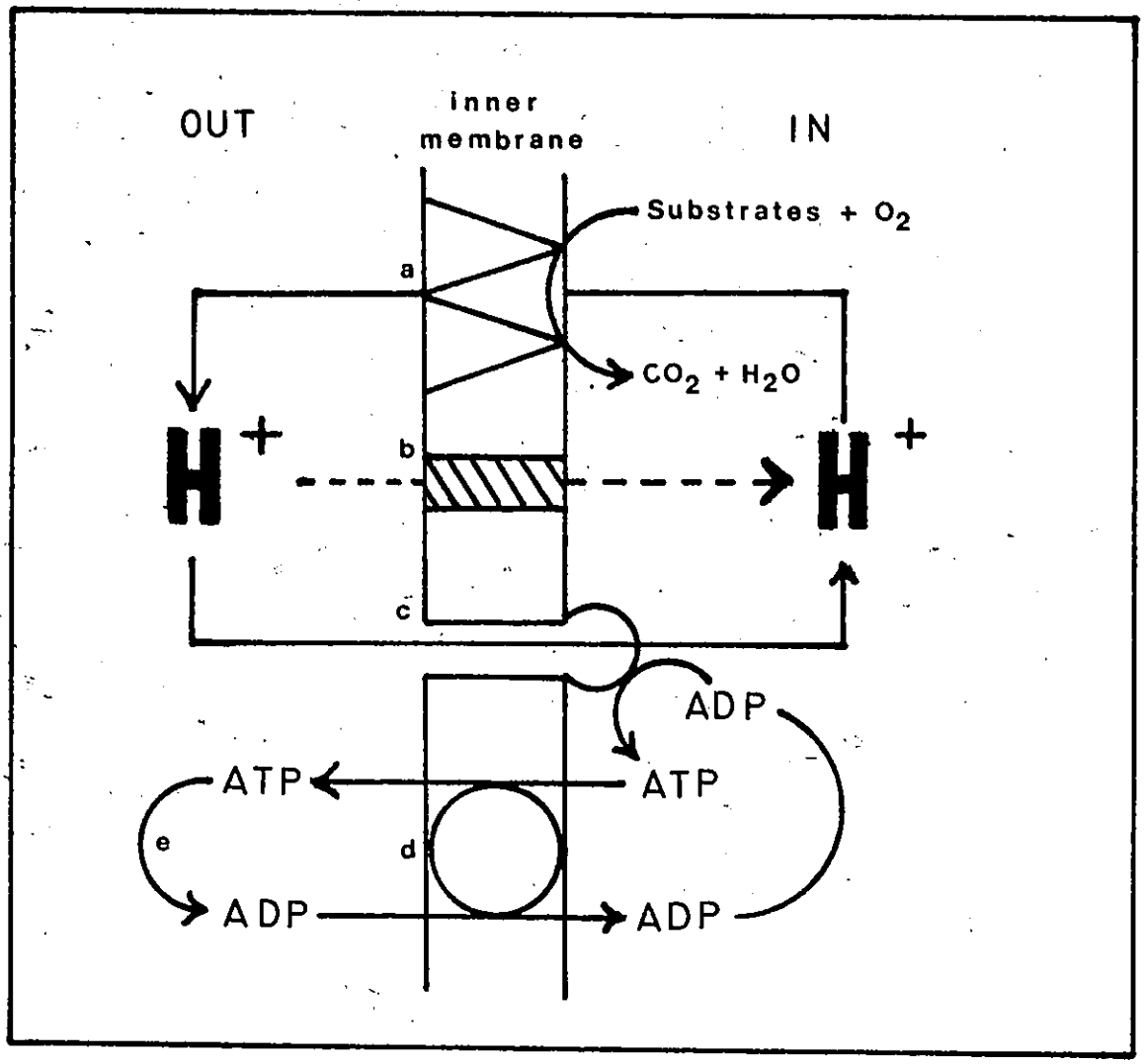

FIG. 5. Schematic representation of an inner mitochondrial membrane where (a) represents the respiratory chain, (b) a proton conductance pathway, (c) the ATP synthetase, (d) the adenine nucleotide translocase and (e) the ATPases.

from the free energy released upon ATP hydrolysis and the role of the mitochondria in heat production would then be reduced. Examples of this ATPase mechanism of heat production are well-known in nature. The classic example is the shivering of the muscles of an animal exposed to cold. Muscle activation leads to ATP hydrolysis and the rate of substrate oxidation in the mitochondria is in turn accelerated by the increased ADP supply. Another process that must also contribute to the increase in heat production during muscle activity, at least to a minor extent, is ion pumping. The restoration of the normal polarized state of the sarcolemma must involve pumping of ions by the Na^+/K^+-ATPase (126). An increase in the activity of the Na^+/K^+-ATPase has also been proposed to explain the increase in heat production upon thyroid thermogenesis (71, 265) and after cold-acclimation in brown adipose tissue (94, 141, 146) and in skeletal muscle (267). Other ATP hydrolysing systems are the so-called futile or substrate cycles in which 2 opposing enzymes (such as fructose 1, 6 diphosphatase and phosphofructokinase) catalyse reactions that on the whole, accomplish nothing but the cleavage of ATP. The F6P/FDP interconversion is important as the main source of heat in the flight muscle of certain insects such as moths and bumblebees (138). The same accelerated substrate cycle has also been found in the muscle of the malignant hyperthermic pig (48) but would appear to be of minor quantitative contribution to the overall thermogenesis in these animals (126). The acceleration of a triglyceride cycle (lipolysis followed by re-esterification) as an ATP consuming process has also been found in brown adipose tissue and proposed as a possible mechanism of NST in CA rats (129). However, there is no strong evidence at the present time that the futile substrate cycles

represent major thermogenic processes in animal tissues.

It can also be seen from Fig. 5 that there are other ways than ATP hydrolysis to increase substrate oxidation and heat production. One way to achieve this, is to uncouple the oxidation of substrate by the respiratory chain (a in Fig. 5) from the normally obligatory expulsion of protons from mitochondria. In other words, it is to prevent the formation of the proton gradient. Examples of this sort of mechanism exist in nature. In some plant tissues, the rate of respiration is high enough to raise the tissue temperature appreciably above the ambient temperature. Such heating occurs in ripening fruit, germinating seeds and opening inflorescences of certain plants but is most marked in the spadix of the Araceae where the increased temperature facilitates the volatilization of compounds that attract pollinating insects (138). The mitochondria from this tissue possess an alternate electron transport pathway (cyanide-insensitive) consisting of the same set of dehydrogenases as the normal respiratory chain, but entirely by-passing the cytochromes via a second oxidase. This alternate pathway is regulated by the activity of the normal cytochrome pathway (6). No such alternate respiratory chain has however been demonstrated in animal tissues.

A third way to increase respiration and heat production would be to dissipate the proton gradient formed upon substrate oxidation (pathway b in Fig. 5). There is always normally a certain recycling of protons consisting of a passive leakage of H^+ ions into the matrix followed by an active extrusion of these ions out of the intramitochondrial space. This has been shown to be an important energy dissipative process giving rise, at least in part, to the state 4 respiration of the mitochondria (269). An increased leakage of H^+ ions back into the matrix leads then

to uncoupled (complete dissociation of ATP synthesis from O_2 utilization as occurs upon DNP or FCCP addition) or loosely-coupled (decrease in ATP synthesis per O_2 utilized) mitochondria. These mitochondria are characterized by a high rate of respiration with no or very little respiratory control. The rate of respiration is then controlled by the availability of substrate for oxidation. Brown adipose tissue mitochondria are loosely-coupled (81). They are unique in possessing a specific and controllable proton conductance pathway (b in Fig. 5) that allows the protons to leak back into the matrix and gives rise to a high rate of respiration (futile proton cycling) (194, 195).

There is an other way of increasing energy dissipation via the active translocation of ions. The accelerated cycling of ions such as Ca^{++} can give rise to loosely-coupled mitochondria. For instance, Na^{+}-induced Ca^{++} cycles are known to occur in heart, brain, adrenal cortex, parotid gland and BAT (2, 29, 51, 52). An example of such a cycle as it is known to occur in heart is given in Fig. 6.

Indeed, the acceleration of Ca^{++} cycles has been proposed as a possible mechanism of uncoupling to explain the catecholamine-induced increase in heat production from skeletal muscle (101) and brown adipose tissue (2, 43, 135).

In conclusion, there are basically two mutually exclusive hypotheses to explain the increase in heat production of CA animals:

- The ATPase hypothesis which requires tightly coupled mitochondria, responding to changes in the phosphorylation state ratio.
- The loose-coupling hypothesis in which the mitochondria are in a loosely-coupled state; their rates of respiration are being controlled by the availability of the substrates.

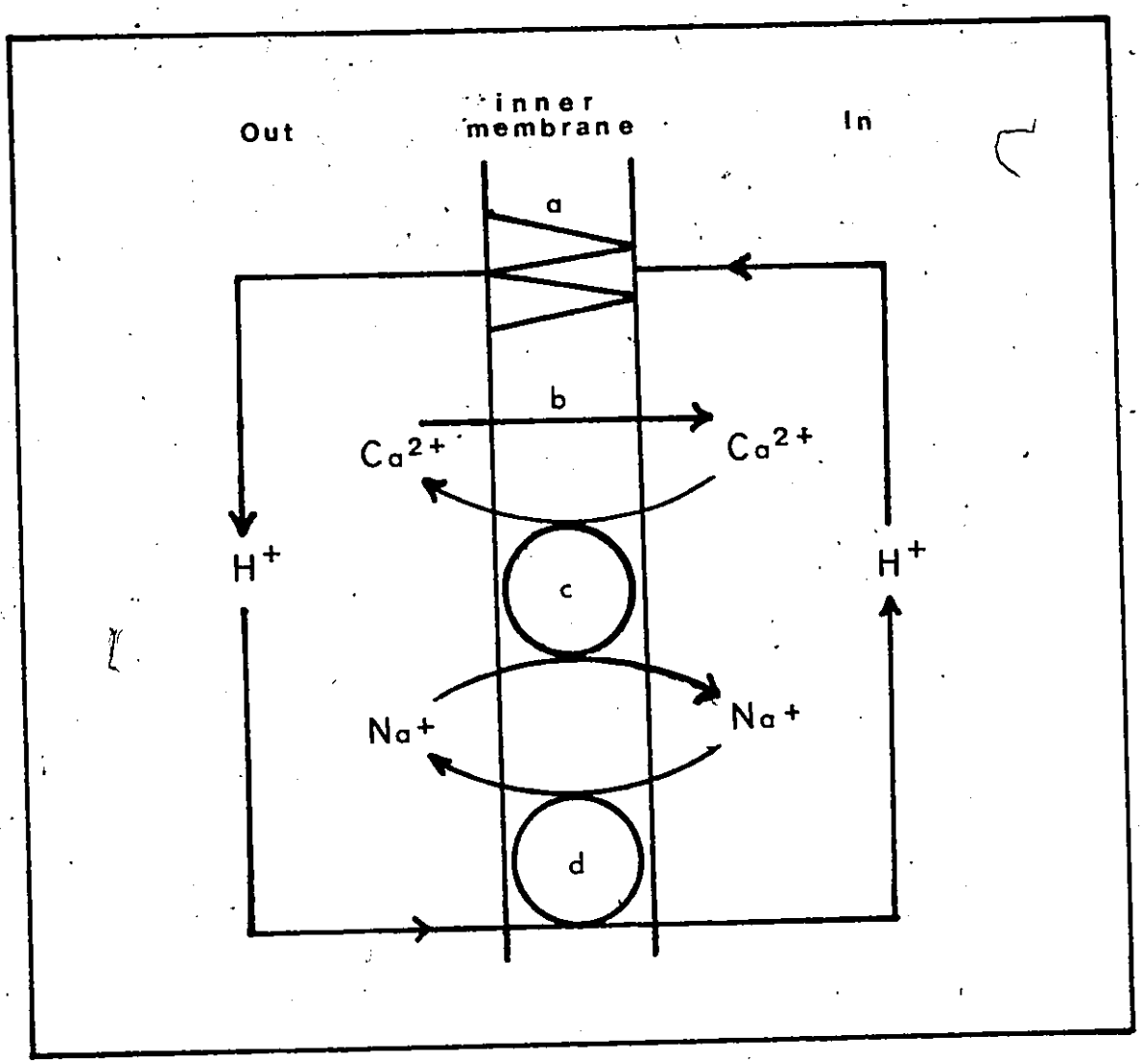

FIG. 6. Schematic representation of the Ca^{++} translocation cycle in heart mitochondria showing some of the metabolite exchange system also located in the inner membrane which influences the Ca^{++} fluxes.

(a) is the electron transport chain, (b) the Ruthenum Red sensitive Ca^{++} carrier, (c) Ca^{++}/Na^+ exchange and (d) the Na^+/H^+ exchange.
(From Bygrave F.L. (29)).

It must also be pointed out that the site of the heat production is not the same, being the cytosol or the plasma membrane in hypothesis A and the mitochondria in hypothesis B.

PART III. BROWN ADIPOSE TISSUE AND NONSHIVERING THERMOGENESIS

A. Structure, composition and function of BAT

As stated earlier, BAT is the main site of heat production in animals exposed to cold. It is the only organ with the main function of thermogenesis. BAT is found in homeotherms and is most abundant in animals having a large capacity to use NST such as newborns, hibernators and cold-acclimated animals. BAT occurs in discrete masses within the body. The most common are the cervical, interscapular, axillary, paraaortic, mediastinal and perirenal (8). They contribute about 0.5-3% of the total body weight depending on the species and the state of adaptation (264). The vascularity of the tissue is extensive (264) and the amount of blood passing through it may take a considerable proportion of the cardiac output (85, 86). Its rich network of blood capillaries, as well as its high cytochrome content contribute to its characteristic brownish color (81). BAT also possesses an extensive adrenergic innervation (49). There are two different sympathetic nerve supplies to the BAT of the rat, one to the blood vessels and the other predominantly to the fat cells (50, 62). BAT is then under neural control and the thermogenesis in the organ is mediated by way of the sympathetic innervation (49, 122, 250). Fully differentiated, BAT is a rather homogeneous tissue. A typical brown adipocyte is a polygonal cell having numerous and large mitochondria, a varying number of lipid droplets and few membranes in the cytoplasm (8). The predominant ultrastructural feature of the BAT is certainly the abundance of mitochondria which appear as particles with a matrix of medium to high density, exhibiting a complex internal structure characterized by numerous, tightly packed and regularly

arranged cristae (81). The mitochondria are distributed throughout the cytoplasm, surrounded by and often in close contact with the numerous lipid droplets, favouring a rapid oxidation of lipids. The number of mitochondria as well as their structure and the number of their cristae varies with the species and the state of adaptation (81).

In the neonatal rat, BAT is present at an advanced state of differentiation characterized by a high rate of respiration associated with a high functional activity of the tissue (8, 10). This period is followed by a regression of the tissue which can be considered to continue for the remainder of the life of the laboratory rat (8). This period of regression may be reversed to the typical post-natal condition by chronic exposure of the adult to cold (264). A series of changes occurs in BAT during these periods of differentiation and regression. These will be reviewed in the next chapters.

Basically, well differentiated BAT is adapted for an aerobic energy metabolism characterized both by a high capacity for fatty acid and triacyl glycerol synthesis as well as a high capacity for fatty acid oxidation (264). BAT has also a high citric acid cycle activity but a low glycolytic capacity (292). This highly aerobic metabolism is the main fundamental difference between BAT and white adipose tissue, the latter, being specialized for the storage and release of fatty acids, although BAT is also capable of exporting fatty acids (18, 33). However, the origin of the BAT, its precursor cells, and whether it can be classified as a completely different tissue or as a more (or less) differentiated form of white adipose tissue is still not clear. In general, the differences between BAT and WAT are more quantitative than qualitative (264). Although there are dissimilarities in structure and

composition (264) and there is an increasing amount of evidence showing differences in their responses to various hormones (19, 74, 154, 173, 260, 262), no single criterion has yet been found entirely reliable to clearly differentiate BAT and WAT. A recent report even shows the in vitro transformation of BAT into WAT (70) and supports the suggestion by Cannon that the classification of all the fatty tissues in two clearly separate groups is a gross oversimplification. She proposes the possibility of these being a spectrum of adipose tissues in which, perhaps, the epididymal white fat and the BAT of the newborn are the extremes (33). Certainly, more research is needed towards the identification of adipocyte precursor cells and their differentiation pattern in order to fully elucidate the problem.

B. Brown adipose tissue mitochondria

In BAT, the chemical energy of the substrate oxidized by the mitochondrial respiration is mostly converted into heat, while in other tissues, it is mostly converted into ATP. BAT mitochondria therefore differ in a number of characteristics from other mitochondria and have been studied extensively (81, 194). There are three major points that will be reviewed here and that are most important with regard to the function of the brown adipose tissue. BAT mitochondria (1) have a very high respiratory capacity, (2) have a very high capacity for fatty acid oxidation, (3) are de-energized upon isolation due to a high proton conductance of their inner membrane.

1. Composition and respiratory capacity of BAT mitochondria

The sedimentation behaviour of BAT mitochondria from guinea pigs is very different from that of liver mitochondria from the same animals (263, 280). The lipid/protein ratio is higher in mitochondria from BAT

than in those from liver (263, 280). In addition, BAT mitochondria from C-A rats have a higher phospholipid/protein ratio than those from W-A animals (280). This is in contrast to guinea pigs where the lipid/protein ratio is similar in the cold- or warm-acclimated states (263). Differences also exist in specific lipid and protein composition of BAT mitochondria from C-A or W-A rats. Chronic cold exposure increases the amount of phosphatidylethanolamine (greatest effect), phosphatidylcholine, cardiolipin and lysophospholipid in the mitochondrial membrane (239). At the same time, there may be alterations in the fatty acid composition of the mitochondrial phospholipids (239) although this is not always observed (34). Variation in diet or strain of animal may explain the discrepancy between these reports.

The mitochondrial membrane of the BAT, compared to that of liver, possesses a very high activity of oxidative enzymes (36, 151, 254) but a low activity of F_1-ATPase (36, 150, 151). It also has a higher content of the respiratory chain components (cytochromes and flavoproteins) (151). There is however no increase in the mitochondrial content of these respiratory chain components upon cold adaptation (226). However, the specific activities of the oxidative enzymes (succinate dehydrogenase, cytochrome oxidase) are higher in mitochondria from cold-acclimated rats when compared to warm-acclimated rats. Their activities actually reach the same value as observed during the early post-natal development of the rat (254). Similarly, the mitochondrial inner membrane content reaches a maximum shortly after birth and then regresses slowly to reach a minimum in the adult life but can be increased maximally, back to its post-natal appearance, upon cold acclimation (10). It appears that there is a limit to which the individual mitochondrion can increase its content of inner membranes. SDS-polyacrylamide gel electrophoresis of mitochondrial

membrane proteins confirms that the relatively larger amount of respiratory components and the relatively smaller amount of F_1-ATPase in BAT mitochondria when compared to liver mitochondria is due to an altered content of the enzymes under study (151). This modified proportion between the ATPase and the oxidative enzymes is consistent with a thermogenic role (energy dissipating) for the mitochondria of the brown adipose tissue. It is inconsistent with the energy-conserving role (ATP production) this organelle has in other tissues such as the liver. This point will be developed further in section III d. The SDS-polyacrylamide gel electrophoresis pattern of mitochondrial membrane proteins from cold-acclimated rats also appears different from the one obtained from warm-acclimated rats. There is one major difference, namely, an increase in a polypeptide of 32 000 M.W. which disappears during readaptation of the animals to a normal temperature (236). This polypeptide has been identified recently as being an important regulator involved in the control of the conductance of protons across the inner mitochondrial membrane (114). There is then no doubt that the metabolism of proteins is altered in BAT and particularly in mitochondria. Further evidence is available, showing a decrease in the half life of certain mitochondrial proteins in cold-acclimated rats (26). More recently, an increase in amino acid incorporation observed in vivo and in vitro has been reported during the acclimation process (27). These changes are absent in non-thermogenic tissues.

2. *Fatty acid oxidation capacity of BAT mitochondria.*

Fatty acids are the main fuel utilized by BAT for thermogenesis. Accordingly, the rate of transport, activation and oxidation of fatty acids by isolated mitochondria are very high. BAT has a long chain acyl CoA synthetase located exclusively on the outer mitochondrial membrane as

opposed to the liver where the enzyme is found in the endoplasmic reticulum and mitochondrial fractions (214). In cold-adapted guinea pigs, the rate of the synthetase activity exceeds by 10-15 times the capacity of the mitochondria for fatty acid oxidation (214). This enzyme is then most probably not a rate limiting step for the respiratory rate of the BAT. The second important enzyme in the chain of events leading to fatty acid oxidation is the "outer" long chain acylCoA transferase in which the activity in BAT mitochondria from cold-acclimated guinea pigs exceeds that of liver by 20 x and is comparable to the activity of the acylCoA synthetase (206). The acyl carnitine/carnitine exchange and the "inner" long chain acylCoA transferase appear to be even more active (206). A high activity of the β-oxidation complex of BAT mitochondria is also evident from the high rate of long chain acyl carnitine oxidation (31, 69, 211). An iron-sulfur (Fe-S) flavoprotein has been identified as a component of the acyl-dehydrogenase complex in brown fat mitochondria (82). There is actually a 5-10 fold increase in mitochondrial Fe-S centers (protein) /wet weight of BAT during cold acclimation but this is due to the large increase in the concentration of the mitochondria within the tissue (82).

Fatty acid oxidation in the mitochondria is dependent on the operation of the TCA cycle. The enzymes of the citric acid cycle in BAT mitochondria appear to be of sufficient activity so that all the acetyl groups generated by the β-oxidation can be rapidly and completely oxidized (226, 227). In the hibernating hamster, however, the body temperature is too low to allow normal functioning of the TCA cycle. Acetate is the end-product of fatty acid oxidation and is formed from acetyl CoA by an active acetyl CoA hydrolase (17). This allows continual fatty acid oxidation by removing the acetyl-CoA formed and thus preventing an end-

product inhibition of the process. BAT mitochondria also oxidize glycerol-3-phosphate very rapidly (25, 226) by means of a flavoprotein-linked enzyme located on the outer face of inner membrane (25). In addition, a cytoplasmic NAD-linked enzyme is also active in BAT suggesting that a glycerol-3-phosphate shuttle for the mitochondrial oxidation of NADH generated in the cytoplasm is quantitatively important in brown fat (149). Thus, the mitochondria of brown adipose tissue have a higher capacity for respiration and fatty acid oxidation than any other non-thermogenic tissue. These capacities are also further enhanced by cold adaptation. They are very well suited for contributing significantly to the main function of BAT, namely, heat production.

3. *Energy state and proton conductance of isolated BAT mitochondria.*

BAT mitochondria, in common with all mitochondria, convert the energy resulting from the oxidation of substrate into a proton electrochemical gradient across the inner membrane (see Fig. 4, section II). During sustained respiration, the rate at which protons are expelled by the respiratory chain is equal to the rate at which they re-enter the matrix. If these rates are unequal, a proton gradient is built up and the respiration is inhibited. Indeed, it has been demonstrated in hamster BAT mitochondria that the rate of controlled respiration decreases linearly as the proton electrochemical gradient increases (Fig. 7).

The rate of respiration depends on the effective proton current going across the inner membrane which is itself dependent on the magnitude of the proton gradient established across the same membrane. The proton conductance of the mitochondria is defined as the effective proton current crossing the membrane per unit of proton electrochemical gradient (199). A large proton gradient results from a low permeability (current) of the

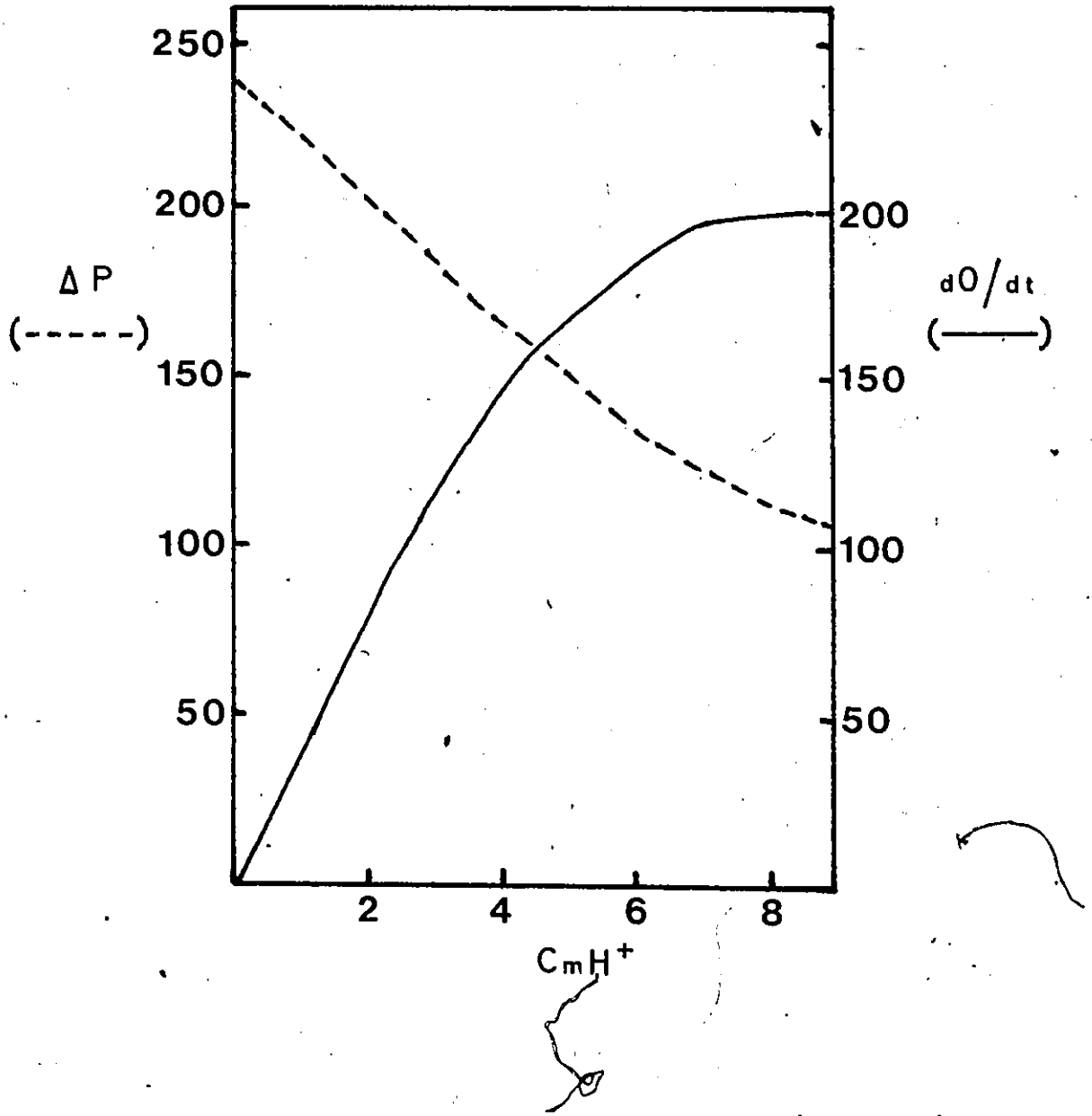

FIG. 7. The proton electrochemical gradient (ΔP, in mV) and the rate of controlled respiration (dO/dt, in nmole O_2 min^{-1} mg^{-1}) of hamster BAT mitochondria oxidizing long chain acyl carnitines, as a function of the effective proton conductance of the inner membrane (CmH^+, in nequivalent H^+ x min^{-1} mg^{-1} mV^{-1}).
(From Nicholls (195))

membrane to protons. The respiratory chain is translocating protons against the proton gradient. A large proton gradient thus, gives rise to a low rate of respiration.

It is possible to measure the proton electrochemical gradient of isolated mitochondria (197). The measurement of this gradient appears to provide a valid indication of the mitochondrial "energy potential". It is also possible to quantitate the energetic state of the mitochondrial inner membrane by measuring the fluorescence activity shown by incubated mitochondria in presence of certain fluorescent dyes (224) or by measuring the degree of reduction of the cytochrome b complex used as an internal probe (81). Using these three methods, it has been found that BAT mitochondria from hamsters or cold-adapted guinea pigs are completely de-energized when freshly isolated (81, 200, 224). They fail to maintain any proton electrochemical gradient during respiration (200). This is also reflected by the respiratory and phosphorylative properties of the mitochondria. Indeed, BAT mitochondria, when isolated and incubated under conditions conventionally used for studies of other mitochondrial systems are characterized by a low P/O ratio, a high state 4 respiration, and little or no effect of ADP, Ca^{++}, uncouplers, or oligomycin (81, 99, 194, 224, 227). They are not however fully uncoupled but rather loosely-coupled since some oxidative phosphorylation is still observed (99) and some Ca^{++} can still be taken up in an energy dependent process (135). In fact, Nicholls calculated that in brown adipocytes, during NST, the mitochondrial oxidative phosphorylation alone would be adequate to maintain the cellular ATP levels (201). The high state 4 respiration could result from an increase in proton re-entry into the matrix or from a breaking of the link between respiration and proton extrusion. The proton extrusion

is however normal in BAT mitochondria and does not differ from that observed in mitochondria from other sources (200). However, hamster BAT mitochondria exhibit an effective proton conductance which is at least a 100 x greater than that of liver mitochondria prepared under parallel conditions (195). The protons are leaking back into the matrix of the BAT mitochondria at a very rapid rate. Normally, protons re-enter the mitochondria via the ATP synthetase, with the formation of ATP. However, in BAT mitochondria the ATP synthetase activity is extremely low (28) and cannot account for the observed respiratory rates. It appears then that BAT mitochondria possess a pathway of proton re-entry which is not coupled to the ATP synthesis. Fig. 8 III depicts the proton circuit as it is thought to occur in BAT mitochondria.

The proton conductance pathway of the BAT mitochondria possesses an effective proton current which is proportional to the proton electrochemical gradient (199). Therefore, any factors that will induce the build-up or the collapse of the proton gradient will then effectively regulate the respiratory rate of the mitochondria. Experimentally, proton entry is not distinguishable from an OH^- exit. From permeability studies, the channel appears to be actually an OH^- uniport (195, 198, 203, 205). Here, to avoid confusion, the term proton conductance will be used. This pathway must however be controllable in vivo to prevent the mitochondria from de-energizing when the tissue is not in a period of active thermogenesis. There are three main factors known to control the rate of respiration of BAT mitochondria (Fig. 9) (200, 224). The rate of respiration is dependent on the pH of the incubation medium, the removal of endogenous fatty acids and the presence of exogenous purine nucleotides. The proton electrochemical gradient of the inner membrane is dependent on the same

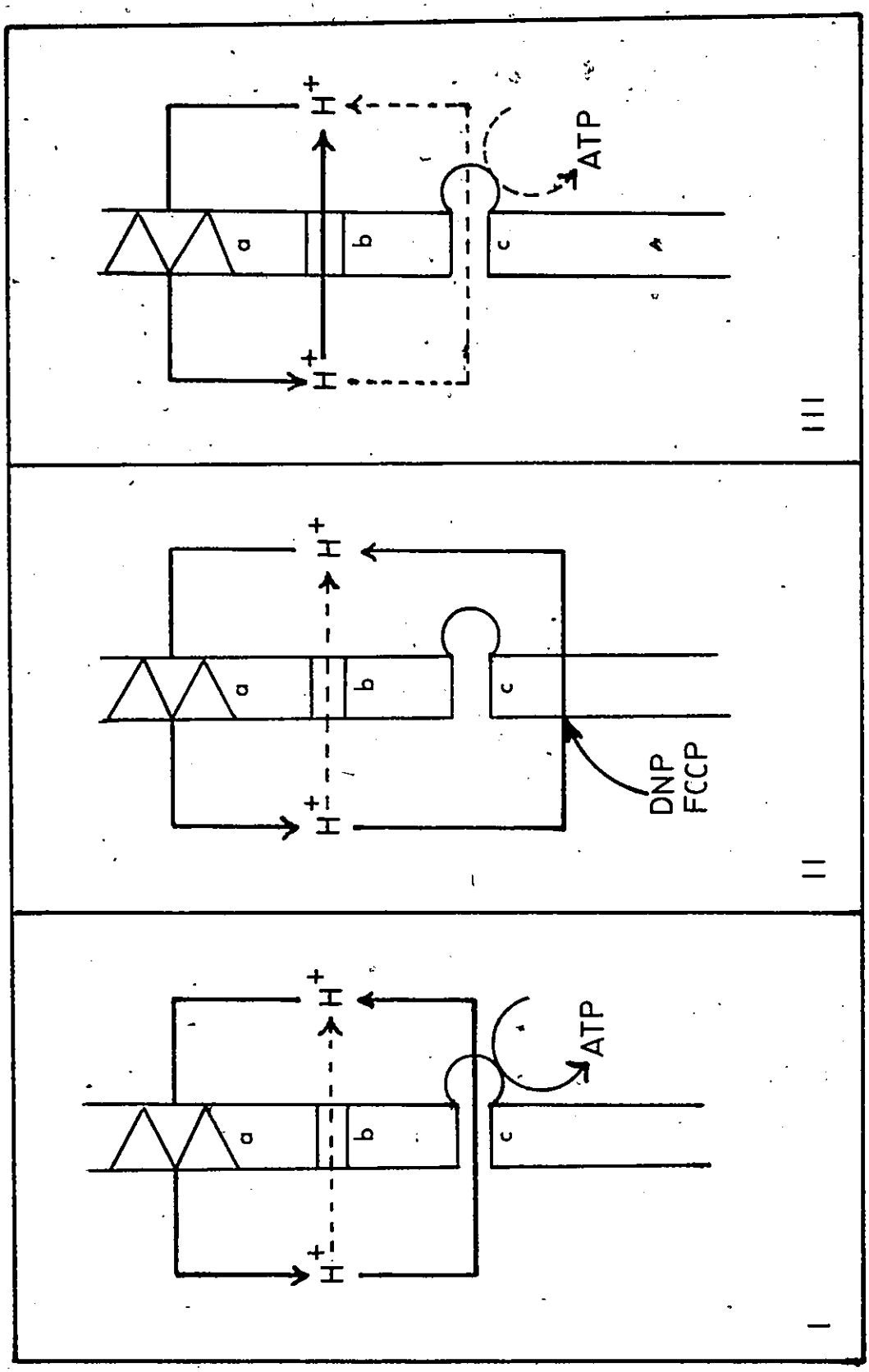

FIG. 8. The proton circuit across the inner membrane of respiring mitochondria where (a) is the respiratory chain, (b) the proton leaking system, (c) the proton translocating ATP synthetase in: I. tightly coupled mitochondria as in the liver
II. uncoupled mitochondria (after addition of FCCP or DNP) or
III. loosely-coupled mitochondria as in thermogenic BAT.
The solid line represents the major proton circuits while the broken one follows minor pathways.

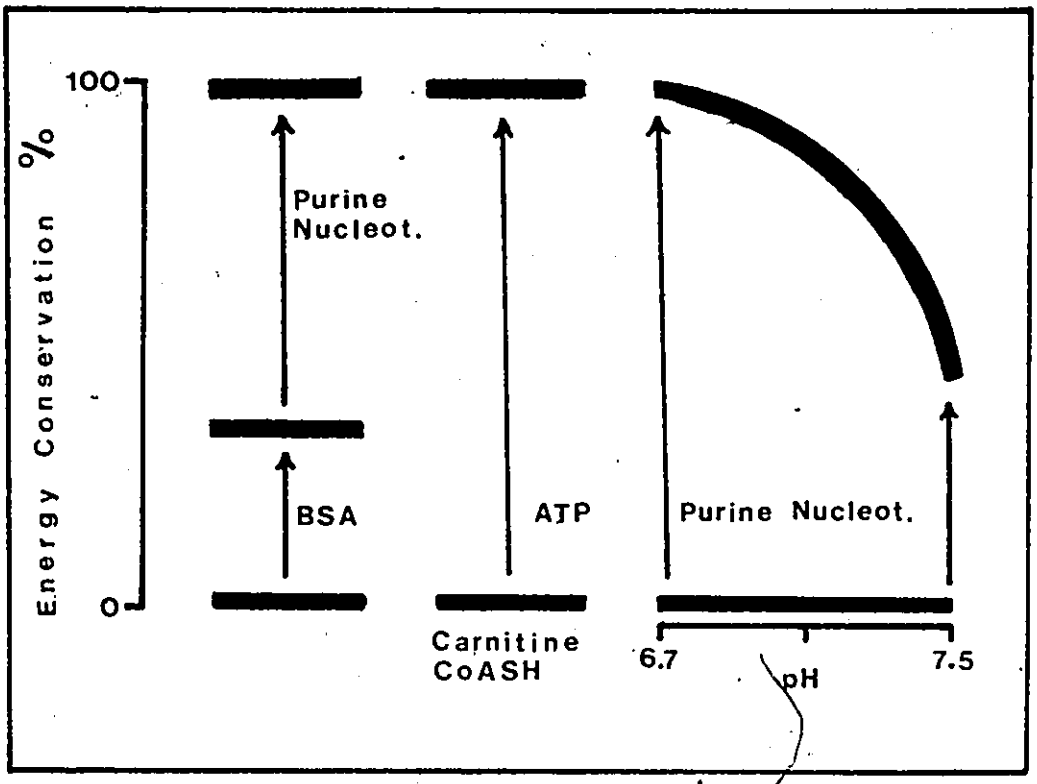

FIG. 9. Alternative factors required to restore the energy coupling in BAT mitochondria. Freshly isolated loosely-coupled BAT mitochondria (0% energy conservation) may be transformed into a tightly coupled state (100% energy conservation) by removing endogenous fatty acids in the presence of exogenous purine nucleotides. BSA: bovine serum albumin. (From Rafael (224)).

parameters (200).

Freshly isolated mitochondria are uncoupled regardless of whether or not the tissue is in a thermogenic state because of the large amount of fatty acids that become bound during the isolation. By careful manipulation of the incubation conditions, it is possible to restore the respiratory control of the mitochondria. The removal of fatty acids recouples the mitochondria. Fatty acids are known to increase the conductance of the inner membrane of the mitochondria and those from BAT appear to be extremely sensitive to their uncoupling effect (112). Fatty acids can be removed by albumin (81, 99) or by oxidation upon addition of carnitine, ATP and CoA (28, 136). Significantly, the degree of recoupling of albumin-treated mitochondria appears to be related to the thermogenic state of the animal. For example, BAT mitochondria from newborn or cold-adapted guinea pigs show a reduced P/O ratio when compared with mitochondria from fetal or warm-adapted guinea pigs (45, 81). However, the recoupling induced by albumin alone is not as extensive as the one observed after carnitine treatment. Only the addition of nucleotides to the albumin-treated mitochondria induces the same degree of recoupling as the carnitine treatment (see Fig. 9). Extramitochondrial nucleotides are then essential to restore the energy conserving system of the mitochondria. Nucleotides effectively modulate the proton permeability of BAT mitochondria. At pH 7.1, the effective proton conductance of freshly prepared hamster BAT mitochondria is greater than 35 nequiv. H^+-min^{-1} -mg^{-1} -mV^{-1} at 23°C. This figure is reduced to 7-10 in presence of albumin, to 2 in presence of nucleotides and to 1 in presence of both albumin and nucleotides (200). Nucleotides inhibit the proton leakage toward the matrix, promote the build-up of the proton electrochemical gradient and, hence, restore the

respiratory control of the mitochondria. Thus, the maximum recoupling induced by the carnitine treatment is due to both the removal of fatty acids and the presence of ATP needed for the activation of the fatty acids.

The interaction of nucleotides with hamster BAT mitochondria has been studied and a specific nucleotide binding site has been identified on the outer face of the inner membrane (196). A whole range of nucleotides can bind to this site and restore the respiratory control of the mitochondria. GTP, GDP, ATP, ADP are very effective. IDP, ITP are somewhat less effective while nucleotide monophosphates and pyrimidine nucleotides are totally ineffective (113, 196). GDP is commonly employed to measure the number of sites because it is not transported into the mitochondria and does not introduce the difficulty of having to differentiate between binding and translocation as is the case for other nucleotides such as ADP or ATP. One peculiarity of the binding site is that all nucleotides capable of inhibiting the proton current of the inner membrane compete with GDP for binding (113). The action of the added nucleotide on the proton conductance of the mitochondria is extremely rapid and freely reversible (113). Only externally added nucleotides are effective and the large amount of nucleotides present within the matrix of the mitochondria is without any effect on the membrane proton conductance (196). The nucleotides bind at a site on the inner membrane different from the adenine nucleotide translocase (196) to a specific polypeptide of 32 000° molecular weight (114, 235). There is a single class of site, sensitive to pH (196). The apparent dissociation constant varies from 4 µM at pH 6.7 to 30 µM at pH 7.9 in hamster BAT mitochondria. This probably explains the in vitro pH dependency of the oxidative phosphorylation of BAT mito-

chondria. It has been reported that the P/O ratio of BAT mitochondria incubated in presence of nucleotides increases progressively as the pH of the medium is lowered (81).

As far as cold adaptation is concerned, several lines of evidence point to the proton short-circuit as being the molecular site of non-shivering thermogenesis in BAT. Some measure of respiratory control is obtained with mitochondria from warm-adapted rats or guinea pigs but not with cold-adapted animals (45, 81). Unfortunately, the energy state of cold- and warm-acclimated animals has not been systematically studied. Proton electrochemical gradients for example, have only been measured in hamster or in cold-adapted guinea pigs without warm-acclimated controls. However, using the redox state of the cytochrome b complex as an internal probe, Pedersen and Flatmark (212) found differences in the energy state of mitochondria from warm-readapted guinea pigs when compared to cold-stressed guinea pigs, depending on the incubation conditions. Significantly, a higher concentration of ATP was needed to obtain half maximum energization in mitochondria from cold-stressed animals than in mitochondria from warm-readapted animals. The importance of the nucleotide-sensitive proton conductance pathway in heat production has been demonstrated recently by direct calorimetry of isolated mitochondria from cold-acclimated rats (234). A large decrease in heat output from the mitochondria was observed after the addition of GDP.

BAT mitochondria from hamster or newborn guinea pigs have a large capacity to bind purine nucleotides (225). In the guinea pig, this large binding capacity is not present during the prenatal period and is lost 10 days after birth (225). The relative amount of the 32 000 polypeptide correlates well with the nucleotide binding capacity of the mitochondria

and with the thermogenic state of the BAT (114, 236). On the other hand, a non-thermogenic tissue such as the liver, has a very low binding capacity for GDP (196). This is consistent with a specific thermogenic role for this unique nucleotide-sensitive proton conductance pathway in the mitochondria of the BAT.

C. Control of BAT thermogenesis on acute cold exposure.

During adaptation of rats to cold, the cellular morphology of the BAT changes markedly. The response of BAT to cold involves two stages:

- an initial rapidly-occurring stimulation of thermogenesis characterized by an increase in blood flow, lipolysis, heat production and heat loss.
- a more gradual and sustained response of the tissue as the cold exposure is prolonged over several days. This phase involves an increase in BAT mass and of its mitochondrial content and is characteristic of the process of cold acclimation.

The changes observed in BAT upon acute exposure to cold are independent of its thermogenic state. In newborns and in cold- or warm-acclimated rats acute exposure to cold (<24 h at 4°C) induces an increase in blood flow through the tissue (86, 117), a depletion of its glycogen and lipid content (270, 271) and an increase in size of its mitochondria (270, 271). The temperature of the brown adipose tissue is maintained above that of the other tissues (152). According to Brück (21), cold stimulates cutaneous thermoreceptors that activate the sympathetic nervous system and lead to NE release in BAT. This activation is controlled by the hypothalamus in which the action is itself regulated by the core body temperature. There is considerable evidence that the acute phase of cold exposure is mediated by NE. The calorigenic response to infused catecho-

lamines is known to occur in BAT (85). Large amounts of catecholamines are secreted by the sympathetic nervous system within BAT in response to cold (58, 59). The temperature of the BAT is also maintained above that of other tissues after NE treatment and this temperature increase is abolished by β-adrenergic blocking agents (54). The ultrastructural changes observed in the tissue after acute cold exposure are mimicked by NE (270, 288) and also abolished by β-blocking agents (270). The recent development of methods for isolating adipocytes from the brown fat has led to a much clearer picture of the mechanism of action of NE. NE stimulates respiration of the brown adipocytes (18, 76, 134, 164, 192, 216, 217, 219, 220, 230, 231) and there is a good agreement between the measurements of the O_2 consumption rate gathered in vitro or in vivo (33). Some sequences of the events occurring after the binding of the catecholamines on the adipocyte membranes and leading to the increase in respiration are now well understood while some others are still obscure. Basically, the response of the brown fat depends on three different sequences of events which are intimately interlinked. The first control is at the level of the plasma membrane and the synaptic space, where the concentration of the NE reaching the cell is regulated, subsequently followed by the binding of the hormone to the membrane receptors. After this step, two phenomena may be distinguished. There is an increase in substrate supply (lipolysis) and an increase in substrate utilization (respiration).

1. Membrane and intersynaptic space events.

Neuronal and extraneuronal uptake followed by the inactivation of NE can modify its concentration in the vicinity of the adrenergic receptors and hence, affect the thermogenic response of the BAT. In vitro, neuronal uptake is able to decrease the concentration of NE in the BAT interstitial

space to at least 1/3 of its concentration in the medium (66). The NE thus taken up into the neurons is either stored in vesicles or catabolized by monoamine oxidase (MAO). The extra-neuronal uptake-catechol-O-methyl transferase system (COMT) seems to play also a significant role in terminating the action of NE (66). This enzyme catalyses the transformation of NE to normetanephrine. In fact, large responses to nanomolar concentration of NE are only observed when both COMT and neuronal uptake are blocked (40, 67). This suggests that such a control of the hormone concentration at the membrane level may be operative even under basal conditions. With respect to the nature of the adrenergic receptors, experiments in vivo as well as in vitro indicate that BAT possesses both α and β-adrenergic binding sites (140, 146). The β-receptor site appears to be of the β_1-subtype of adrenergic receptors (isoproterenol > NE ≥ E)(24). Both α and β-adrenergic agonists appear capable of stimulating brown fat heat production (134, 140), although the contribution of the β-pathway may be quantitatively more significant (23, 217). However, there is a difference between the α or β-adrenergic stimulation as regard to their effect on lipolysis. Isoproterenol, a β-agonist, enhances glycerol release from brown adipocytes while phenylephrine, an α-agonist, does not (140). This may explain, at least in part, the greater calorigenic response of the cells to the β-agonists.

The first observed effect in vitro (94, 252) or in vivo (147) of the binding of catecholamines to the brown adipocytes is a depolarization of the plasma membrane. A sudden change in Na^+ and K^+ permeability has been proposed to explain this depolarization (94) which occurs prior to the stimulation of O_2 consumption of the brown fat (252). Cyclic AMP does not mediate this NE-induced depolarization (166). Depolarization is

also obtained after electrical stimulation of the nerve innervating the interscapular brown fat (147, 252) or by intravenous infusion of NE, isoproterenol or phenylephrine (78, 80). Propanolol, a β-antagonist, at a dosage sufficient to abolish the temperature increase of the BAT in vivo inhibited by 55% the depolarization upon NE administration (147). However, the relation of the depolarization of the cell membrane to the subsequent enhancement of lipolysis and respiration is still unclear. For instance, it is possible to get an increase in respiration without the depolarization with a low amplitude electrical stimulation of the nerve supply to BAT (251).

2. *Substrate mobilization.*

Simultaneously or just after the depolarization, there is a rapid but transient rise in cAMP (134, 217, 257) and cGMP intracellular concentrations (257). Part of the rise in cGMP may be brought about through an indirect mechanism. In white fat, fatty acids are known to activate the guanylate cyclase and could possibly be involved in the indirect stimulation of the guanylate cyclase by β-adrenergic stimulation in brown fat (257). The cAMP increase is mediated through the β-adrenergic receptors (217, 257) while the cGMP level is under α-adrenergic control (257).

The rise in cAMP in turn activates specific protein kinases (256, 162). In vivo, the activation of protein kinases is observed on administration of NE and is also significantly high at birth and on acute cold exposure (256). The activation of a protein kinase is thought to result in a subsequent activation of triglyceride lipase. Indeed, lipase activity correlates well in tissue fragments with the protein kinase activity (256) although, at the present time, no specific triglyceride

lipase has been identified in BAT. However, there is no doubt that lipolysis is greatly stimulated in brown adipocytes after NE addition as measured by glycerol (76, 230, 231) or fatty acid release (18, 76, 192, 230, 231). These measurements may however underestimate in some instances the real rate of lipolysis since there are three possible fates for the fatty acids released from the breakdown of triglycerides in the BAT. The free fatty acids may be re-esterified, be exported outside the cell or enter the mitochondria for combustion. Simultaneous enhancement of both lipolysis and re-esterification has been suggested as an energy dissipating mechanism in BAT (129) and re-esterification of fatty acids is actually enhanced by cold acclimation (129). Both glycerol kinase and glycerol-3-phosphate dehydrogenase are very active in BAT (25, 161). However, it appears that the lipolysis-re-esterification futile cycle would not be sufficient to account for the elevated rate of O_2 consumption occurring during thermogenesis (161, 179). Fatty acid export may also be regarded as physiologically important (18, 192). During NE-stimulated lipolysis in isolated brown adipocytes, the level of cell-associated free fatty acids remains constant or decreases gradually (but does not increase), while the concentration of extracellular fatty acids increases linearly (18). These exported fatty acids are presumably important as substrates for other tissues during active thermogenesis. Finally, and of most importance, fatty acids produced upon NE administration may enter the mitochondria for their subsequent combustion. As mentioned in the preceding section, the mitochondria are very well geared for the combustion of fatty acids. However, in isolated adipocytes, there is a need to maintain a certain energy level in order to get maximum fat oxidation. A high ATP level is needed for the activation of fatty acids

prior to their transport and oxidation in the mitochondria. A decline in ATP level (with concomitant rise in ADP and AMP levels) upon NE addition to brown adipocytes has a negative effect on the respiratory rate when endogenous fatty acids are oxidized (217). It is possible to avoid the decline in ATP level and the subsequent inhibition of adipocyte respiration by bubbling the buffer with CO_2 prior to the addition of cells and NE (216). This beneficial effect of CO_2 is caused by its participation in the carboxylation of pyruvate to yield oxaloacetate in order to increase the level of citric acid cycle intermediates necessary for the rapid oxidation of fatty acids (33). In the brown fat cell, ATP is produced from 3 main sources: glycolysis (179) mitochondrial substrate-level phosphorylation (33, 228, 229) and by oxidative phosphorylation (194). The relative contribution of these processes to ATP production as well as the relative demand of the various ATP utilizing reactions are not clearly established. This, however, represents the central problem in the understanding of the mechanism of thermogenesis in BAT.

3. Substrate utilization and energy dissipation.

NE stimulates respiration of brown adipocytes. The rate of heat production by the BAT is proportional to the mitochondrial fatty acid oxidation. But, concurrently with the enhancement of the mobilization of substrates, NE must also increase the rate of energy consumption of the cells. There is still controversy about the way NE does so. Two hypotheses are proposed to explain the action of NE on the cell respiratory rate: the Na^+/K^+-ATPase and the loose-coupling hypothesis.

The plasma membrane Na^+/K^+-pump has been proposed as playing a major role in brown fat thermogenesis (143). According to this hypothesis, the NE-induced increase in O_2 consumption (and heat production) is due to

an increased activity of the Na^+/K^+-ATPase. The possible involvement of this enzyme in BAT thermogenesis is suggested by several lines of evidence. There is no doubt that NE induces a depolarization of the brown adipocyte membrane and that there is a redistribution of ions, particularly Na^+ and K^+, associated with it (80, 93, 94, 251). This occurs prior to the increase in heat production or in respiration of the BAT and the ion distribution is restored shortly after the removal of the stimulus (nerve electrical stimulation or NE administration) (147). However, as mentioned earlier, it is also possible to obtain an increase in respiration without depolarizing the cell membrane (251). Nevertheless, the key experiments supporting the Na^+/K^+-ATPase hypothesis make use of the inhibition of the activity of the pump by ouabain (specific inhibitor) or by lack of Na^+. A 60-66% decrease in NE-induced increase in respiration is obtained by the incubation of normal (143) or FCCP-uncoupled (208) isolated adipocytes with ouabain. The O_2 consumption elicited by either isoproterenol or phenylephrine is also decreased by 70% in the presence of ouabain (140). A direct stimulation of the Na^+/K^+-ATPase by NE, through cyclic AMP, has been shown on membrane fractions isolated from the BAT of cold-acclimated rats (120, 144). However, it must be pointed out that ouabain has a lot of variable and secondary effects. Not only is there a marked alteration of the intracellular ion concentrations after ouabain binding, but this compound has also an inhibitory effect on enzymes such as adenylate cyclase (137). An alteration of glucose uptake in some tissues is also observed (88). Thus, respiration may be altered by ouabain because of a change in an energy-requiring process other than the Na^+ pump. Moreover, Chinet and co-workers (41) measured the energy expenditure due to active sodium-

potassium transport in BAT of the rat; the Na^+/K^+-ATPase (as assessed by ouabain binding) appears to be only a minor contributor to the overall thermogenesis. There is at present no report comparing the actual activity of the Na^+/K^+-ATPase in BAT of cold- and warm-acclimated animals.

Other arguments can be presented against a major role for Na^+/K^+-ATPase in BAT thermogenesis. BAT mitochondria possess a low content of F_1-ATPase and a high content of respiratory chain components when compared with liver mitochondria (see section III c). In addition, no difference in the activity of the adenine nucleotide translocase is observed between warm- and cold-exposed (6-10 days) guinea pigs (44). It would be expected that a tissue with a large demand for cytoplasmic ATP would have high rate of translocation of adenine nucleotides. The translocase activity of the BAT mitochondria appears similar to that of liver mitochondria (44, 183, 293). In heart, a tissue with a continuous and high demand for ATP, the translocase activity as well as the number of translocase sites, are 3-4 x higher than those of liver mitochondria (160). The Na^+/K^+-ATPase hypothesis requires tightly coupled mitochondria in which the ATP production and transport towards the cytosol need to be maximum. The brown fat mitochondria simply do not seem to be fitted to fulfil this function. This is not to say that the activation of the Na pump upon NE administration is of no importance. This pump is certainly the site of some heat production but its contribution to overall thermogenesis appears to be minor. However, other plausible functions for the enhanced activity of this pump have been proposed. For instance, the activity of the Na^+/K^+-ATPase may be responsible for the generation of an intracellular signal that alters the mitochondrial

coupling in BAT (141), possibly via its alkalinizing effect on the cytoplasm (42).

The experimental evidence presented so far (see Section 3) supports the view that mitochondrial loose-coupling is a major thermogenic process in BAT and that the nucleotide-sensitive proton conductance pathway represents the molecular site of nonshivering thermogenesis. At the cellular level, the mitochondria appear to be loosely-coupled. Brown adipocytes stimulated by NE gradually lose their respiratory control, as demonstrated by a diminishing response to FCCP, an uncoupler of mitochondrial respiration, promoting the re-entry of protons into the matrix (219). However, when the respiration of the cells is maximally stimulated by NE, an inhibitory effect of oligomycin addition (which blocks mitochondrial ATP production) is still apparent. The fact that oligomycin can inhibit the increase in respiration (219) and lipolysis (75) induced by NE indicates that the mitochondria of the NE-stimulated tissue are still capable of producing the ATP needed for the activation of free fatty acids. In vivo, the effect of DNP (an uncoupler) was studied in cold-acclimated rats treated with sufficient NE to elicit a maximum temperature response from the brown fat (145). Following the thermogenic stimulation by the injection of NE, the subsequent injection of DNP still produced a small increase in the temperature of the tissue. Therefore, the mitochondria are not uncoupled, but rather loosely-coupled in the BAT, even after maximal stimulation by catecholamines.

While there is good evidence that cyclic AMP is the second messenger for the stimulation of lipolysis, the nature of the messenger regulating the coupled state of the mitochondria is still unknown. The messenger involved must be capable of both activating proton conductance

during induction of thermogenesis, and by its removal, inhibiting it at its termination. Purine nucleotides, fatty acids, acyl CoA and an intracellular increase in pH have all been proposed as second messengers for NE-induced thermogenesis.

Purine nucleotide concentrations do not change sufficiently on NE addition to be able to modulate the conductance of the mitochondrial proton conductance pathway directly in brown adipocytes (217). In addition, the hydrolysis of ATP to ADP occurring after NE addition (217) would not make any difference as far as the regulation of the proton conductance is concerned since ADP is almost as potent as ATP, at least in vitro, to induce the recoupling of the mitochondria (196).

Fatty acids have been proposed as the physiological modulators of the proton conductance (23, 220). Addition of fatty acids (palmitate or oleate) to isolated brown adipocytes stimulates the respiration of those cells to the same extent as does NE (23, 220). The fatty acids also elicit this calorigenesis at physiological concentration even when β-adrenergic receptors are blocked by propanolol (23). Fatty acids can actually influence the respiratory rate of adipocytes in several ways. They may act as substrate, as ATP consumer (in their activation to CoA esters) or as uncoupler of the mitochondria. BAT mitochondria are extremely sensitive to the uncoupling effect of fatty acids (112). There is a 30 fold greater increase in the effective proton conductance for each nmole of palmitate bound to BAT mitochondria than in liver mitochondria (112). Isolated mitochondria from the BAT contain a substantial amount of bound fatty acids. Some of them, however, originate during the preparation of the mitochondria (28, 112). The elimination of the bound fatty acids alters some of the respiratory characteristics of isolated

mitochondria as well as their morphology (28, 112). However, mitochondria in tissue sections from NE-treated or cold-exposed newborns are swollen and have an expanded matrix (270, 288) while fatty acid treated, uncoupled mitochondria are characterized by a condensed conformation (28). In addition, isolated resting brown adipocytes contain a substantial amount of free fatty acids which does not appear to change upon NE stimulation (18).

Thus, fatty acids themselves as physiological messengers fail to account for the regulation of the specific nucleotide-sensitive proton conductance pathway present in BAT mitochondria. But their CoA derivatives are known to decrease GDP binding to hamster BAT mitochondria and to increase their chloride permeability (35) and the acyl CoA derivatives of the fatty acids have been proposed as physiological messengers (35). They are believed to act as "antinucleotides", competing with the purine nucleotides for the binding sites. Their effect appears to be of an allosteric nature (112). Basically, the hypothesis proposed is the following: NE binds to the cell membrane and activates cyclic AMP production and lipolysis. The resulting increase in the level of fatty acyl CoA displaces the purine nucleotides from the inner mitochondrial membrane and gives the signal for the enhanced proton cycling. As a consequence, the respiration increases. This hypothesis is attractive since it is self-regulatory. The rate of respiration would be controlled by the increased or decreased level of the fatty acyl CoA upon NE addition or removal. The fatty acyl CoA would then act both as the substrate and inducer of the thermogenesis. However, the effect of acyl CoA on ion permeation in BAT mitochondria is only apparent at extremely high acyl CoA levels which cannot be generated enzymatically in the cell and which

lead to a general increase in ion permeability not only in BAT but also in liver (194). This lack of specificity argues against a possible role in the control of thermogenesis in BAT.

Another way of displacing the purine nucleotides from the inner membrane is by increasing the intracellular pH. The mitochondrial purine nucleotide binding is extremely pH sensitive (196). It has been suggested that increased energy dissipation in the mitochondria of BAT might be started by an intracellular alkalinization which would decrease the affinity of purine nucleotides for the proton channel and thus increase proton conductance (42). The action of intracellular alkaline pH may also be indirect by enhancing lipolysis and formation of acyl CoA (42). However, direct evidence that NE can actually induce a rise in intracellular pH has not been obtained so far. There is also no evidence that a small pH perturbation can quickly influence the mitochondrial respiration in the intact cell.

It appears that the main difficulties in identifying the physiological mediator of NE come from the tight relationship between lipolysis and respiration and the impossibility of dissociating one from the other. Another difficulty is the lack of information about the actual state of coupling of the mitochondria in vivo or in the isolated adipocytes. At the present time, it has not been possible to demonstrate directly the mitochondrial loose-coupling effect of NE in intact BAT cells. Most probably, the recent development by Nicholls (139) of a method allowing the determination of the mitochondrial proton motive force directly in isolated cells will prove to be the ultimate tool in finding the answer to this question.

D. BAT and chronic (long-term) cold exposure

The response of BAT to chronic cold has been studied in detail (240, 254, 264, 271, 279). The brown fat mass increases due to hypertrophy and hyperplasia of the tissue and there is an increase in water, protein and DNA content of the tissue. BAT from CA rats also has a lower lipid concentration (129) and smaller but more numerous lipid droplets are apparent within the tissue. The general metabolism of the tissue is activated in CA animals (128, 264). In vitro, respiration with intermediates of fat metabolism such as α-glycerophosphate and β-hydroxybutyrate is enhanced (38). Lipid turnover and glycerol kinase activity are increased (266, 282). Cold treatment also increases the phospholipid content of the tissue and alters the fatty acid pattern of these phospholipids (238). These phospholipid changes reflect the mitochondrial development very much enhanced by cold acclimation. The specific activities in BAT homogenates of certain important mitochondrial enzymes such as cytochrome oxidase, succinate dehydrogenase, malate dehydrogenase, aconitase, etc, are also increased (11, 279). Indeed, exposure of rats to cold induces a large proliferation of mitochondria within the tissue. There is also an increase in the number of cristae, so that the mitochondria now appear as elongated particles with tightly packed parallel cristae. The process of cold adaptation most particularly, appears to be associated with a selective increase in the 32 000 MW nucleotide binding polypeptide in the inner membrane of the mitochondria (114, 236) and with the concomitant induction of the nucleotide-regulated thermogenic proton short-circuit. The response of BAT to chronic cold is then characterized by an increase in the mass of the tissue and its mitochondrial content and an increase in the capacity of the mitochondria for heat production. In

parallel with these changes there is also an increase in the vascularity and innervation of the tissue. The BAT of a cold-acclimated rat thus possesses a greatly enhanced capacity for lipid oxidation, catecholamine-stimulated respiration and heat production. In CA rats, the tissue appears to be in the same adaptive state as in the newborn and all the changes slowly regress when the acclimated animals are returned to the warm.

At the level of the plasma membrane, little information is available. A reduction (189), no change (258) and an increase (13) in the catecholamine-stimulated adenylate cyclase activity have been observed. During cold acclimation, the growth of the tissue is also accompanied by a 3-5 x increase in the total number of β-adrenergic receptors (13, 24) although this increase does not keep pace with the increase in BAT cellularity. An actual 41% reduction in β-adrenergic receptors / cell is observed (24). Upon cold acclimation, there is also a decrease in insulin binding to the BAT membranes (13).

E. Control of BAT growth and composition

The thermogenic function of BAT is controlled by NE released from the sympathetic nervous system upon cold exposure. In cold-acclimated rats, the increase in the calorigenic response to circulating NE is attributed to a greater amount of BAT and to a higher calorigenic capacity per gram of the tissue (60). But is the growth of BAT, its altered composition and its maintenance in the cold-acclimated state also under the action of NE or of some other hormones? A recent report shows that the mitochondrial increase in size and in the number of cristae is greater in intact brown fat of cold-acclimated mice than in denervated brown adipose tissue (261). This study also shows a decreased capillary bed in denervated BAT. This implies then a direct action of NE in the mainte-

nance of the brown fat structure and/or the action of some "factors" coming from the blood stream. Basically, the problem is to identify the hormone(s) responsible for the growth, modification and maintenance of the BAT upon cold exposure. Two ways are being currently used to approach the problem.

The first approach consists of comparing the time course of the changes in the levels of circulating hormones with the time course of the changes occurring in BAT during cold acclimation to see if there is not any correlation existing at this level.

This approach has not been very fruitful so far, mainly because of a lack of information concerning plasma hormone levels during long-term adaptation to cold. Virtually all hormone levels are affected by cold exposure. Upon acute cold exposure, the levels of NE (46, 124), E (46, 124) and TSH (68, 79, 291) rise while glucagon (168), growth hormone (12, 72), MSH (286) decrease. A transient increase in the release of ACTH (286) is also observed as well as a transient decrease in insulin release (12). When the exposure to cold is prolonged over several days, the NE level remains elevated (46, 124) while E returns to normal (124) or remains elevated (46). Plasma TSH levels are increased (163) or unchanged (7). Glucagon level is high after two weeks of cold exposure but is not much altered by the fourth week (168, 169). Changes in thyroid hormones are more complex. There is an increase in thyroid activity upon cold adaptation (89, 268) but also a marked increase in the clearance of T_4 from the plasma through the gastrointestinal pathway (89) so that there is no actual change in the plasma level of T_4 (89, 191). Plasma level of T_3 increases upon acute cold stress or two week long cold exposure (15, 16, 115, 232). The actual determination of rT_3 is

also needed in order to establish clearly the role of thyroid hormones in cold acclimation. It appears then, that from hormone levels alone, and because of a too large variety of species of animals used and of different lengths of time of cold exposure, there is no strong evidence in favour of one hormone or another as an agent for the transformation of BAT, with the possible exception of NE.

However, the removal of various endocrine glands leads to some conclusions. For instance, thyroidectomized or adrenalectomized animals die rapidly of hypothermia upon cold exposure. It is important to note at this point that the lack of survival of such operated animals has no significance as far as BAT is concerned, since various other factors such as impairment of the blood flow or mobilization of substrates may be responsible for their inability to survive a cold stress. For example, adrenalectomized, cold-exposed rats require glucocorticoids for catecholamine-mediated mobilization of free fatty acids, for shivering responses, and for vasoconstriction and piloerection (55). However, the cold adaptation of animals deprived of certain hormones points to a non-essential role of these hormones. In this regard, adrenalectomized rats (with a replacement dose of glucocorticoids) (124) as well as alloxan-diabetic rats (218) can become cold-acclimated, demonstrating the non-essential role of E and insulin. It is not to say that they do not play any role in cold adaptation but that it is a dispensable one. In addition, insulin is known to stimulate lipid deposition in BAT (19, 264) and is unable to prevent the in vitro transformation of BAT into WAT (70). Thyroidectomized rats (with a small replacement dose of thyroxine, they are said to be hypothyroid) can survive in the cold almost indefinitely demonstrating that elevated levels of thyroid hormones are not essential

to cold adaptation (248, 249).

The second approach to the problem of identifying the hormone(s) responsible for BAT growth and changes in its mitochondria during adaptation to cold consists of injecting chronically various hormones in order to reproduce or "mimic" the effect of cold on the BAT. Chronic administration of NE+T_4 into young WA rats induces an increase in the BAT mass, a proliferation of its mitochondria, an increased calorigenic response of the rats to catecholamines and a higher resistance to cold stress (116, 176). Moreover, IBAT is smaller in alprenolol-treated rats than in the controls (109). It is not known whether the effect of NE on the brown fat mass is indirect, mediated via some other hormones, or if NE itself can actually stimulate the differentiation of some precursor cells. NE itself does not stimulate the rate of protein synthesis in isolated adipocytes (33). The control mechanism at the cellular level of brown fat growth may involve cGMP. Skala and Knight (257) found an interesting correlation between the brown adipose tissue content of cGMP and cGMP-dependent protein kinases and the proliferation of the tissue in the rat. The tissue content of cGMP is low in adult WA rats but there is a 5-6 fold elevation in newborn and cold-acclimated rats. They suggest a possible involvement of this nucleotide in the stimulation of the growth of the tissue. cGMP is also known to be involved in the proliferation of various other tissues. Most interesting is the fact that the cGMP content appears to be under α-adrenergic control suggesting a possible involvement of catecholamines acting on α-adrenergic receptors in the control of the growth of the BAT. However, the NE-treated animals are not as resistant to cold as the C-A animals. Possibly, the injections of hormones with short half life, such as NE, may not mimic perfectly

the effect of cold where NE is secreted at high rate on a continuous basis. The best growth stimulation is obtained when both NE and T_4 are administered together. However, when alprenolol is given together with thyroxine, it does not antagonize the metabolic changes due to hyperthyroidism (109). Indeed, the changes obtained in BAT after thyroid administration alone differ from those observed after cold adaptation (116, 173, 238, 264). There is an elevated lipid content (TG) of the tissue not obtained in C-A rats and the phospholipid composition of the mitochondria differs from that seen after cold acclimation (237). Thyroid hormones nevertheless appear to play a significant role in the response of BAT. The increase in the in vitro rate of O_2 consumption of the tissue taken from C-A mice is not observed if the mice have been chemically thyroidectomized (153). Similarly, the rise in tissue O_2 consumption following addition of NE in vitro is not observed in BAT removed from hypothyroid mice (153). It appears that the plasma concentrations of NE and E depend on the thyroid status of the animal (46, 247) and that the ability of hypothyroid rats to acclimate to cold is dependent upon an increased activity of the sympathetic nervous system (248). In addition, thyroid hormones are known to influence the number and the responsiveness of β-adrenergic receptors. Thyroidectomy (surgical or chemical) decreases the number of β-adrenergic receptors in rat white fat cell membranes (95) and the responsiveness to acute administration of isoproterenol (a potent β-adrenergic agonist) (87). Thyroxine treatment, on the other hand, increases the number of β-receptors in white fat cell and heart membranes (47, 159). In heart, thyroxine also appears to have an effect on the α-adrenergic receptors (253). In conclusion, thyroid hormones are necessary to permit a normal thermogenic response

to cold but the relation between thyroid hormones and catecholamines during cold exposure is very complex and certainly more research is needed in order to elucidate their role. The role of thyroid hormones in the growth of BAT does appear to be of an indirect nature.

TSH has also been proposed as a possible regulator of BAT growth by Doniach (68) from observations on patients suffering from untreated congenital hypothyroidism, athyrotic cretinism and adult myxoedema in which the plasma levels of TSH are elevated. Those patients present peculiar fat pads in area of the body known to contain brown adipose tissue.

Brown adipose tissue is also known to be a glucocorticoid target organ. It possesses glucocorticoid receptors (77) and the chronic administration of glucocorticoids induces an hypertrophy of the tissue (173, 264). The lump at the back of the neck of people suffering from Cushing's syndrome is likely due to the hypertrophy of BAT induced by an excess of circulating glucocorticoids. However, the increase in brown fat mass in glucocorticoid-treated rats results from an accumulation of lipid within the tissue. There is no increase in non-fat material (173). In addition, the in vitro increase of O_2 consumption following the addition of NE to BAT slices from control animals is abolished by cortisone treatment (255). Nevertheless, glucocorticoids are known to have a "permissive" effect on the metabolic actions of catecholamines. In developing rats (10 day old), a single injection of cortisone results in an impaired ability of the mitochondria of the BAT to oxidize fatty acids but is without effect on 30 day old rats (255). One to three injections of cortisone to young W-A rats has a deleterious effect on the mitochondrial structure (107). But possibly, the most

interesting finding is that glucocorticoid-treated rats (5-7 days) have a 2 fold increase in the blood flow through their brown adipose tissue (170) as measured by the Sapirstein's method ($^{86}Rb^{+}$ uptake)(170). More accurate determinations of the blood flow through the tissue with the microsphere method is certainly needed before any definite conclusions are to be drawn. It is likely that glucocorticoids exert their effect on brown adipose tissue by interacting with the amount of potential substrate (lipid) through their regulatory control on lipid storage and breakdown and by interacting with the O_2 supply and heat loss of the tissue through changes in blood flow.

The photoperiod also seems to have an effect on BAT. The brown fat mass is increased in C-A rats living in a short photoperiod day (9L:15D) when compared with C-A rats living with a long day period (15L:9D) (106). Short day period also induces a larger NST response to NE in C-A rats but a smaller one in W-A rats. Injection of melatonin is without effect under all conditions in the rat (106). This is in contrast to hamsters where injections of melatonin stimulate growth of brown adipose tissue (118). The effect of the photoperiod on the BAT is then probably indirect in the rat, possibly resulting from the modification of the feeding habit of the animals and consequently the changes in the levels of some factors other than melatonin.

At the present time, no single hormone can be designated as the "causal" agent for the changes in the thermogenic capacity of the brown fat after cold acclimation. The long term response of BAT may reflect the influences of several hormones. There is no doubt that the sympathetic nervous system as a whole is involved in the development of the cold-adapted state. Although available evidence points to its possible

involvement in the control of the growth, composition and maintenance of brown adipose tissue, its precise role still remains obscure. Thyroid hormones and adrenal cortical hormones also play significant roles in mediating the effect of chronic cold although their actions in the stimulation of the brown fat growth are most certainly indirect. It is this tight interrelationship between the various hormonal controls and the capacity of the animal to adapt to various imbalances that make the problem of regulation of metabolic processes so fascinating and, at the same time, so difficult experimentally.

PART IV. SKELETAL MUSCLE AND NONSHIVERING THERMOGENESIS

Although in small mammals the contribution of skeletal muscle to NST appears to be minor, changes occur in this tissue upon cold-adaptation that certainly contribute to the overall thermogenesis. Indeed, the calorigenic response of leg muscles from C-A rats differs from that one of W-A animals (103). Although the increase in the O_2 consumption in response to administered NE is initially the same in C-A and control rats, the limbs from the C-A rats are able to maintain the elevated steady state O_2 consumption for a longer time whereas a decrease is observed for the control rats (103). However, a more direct demonstration of an enhanced calorigenic response to NE in this tissue has not been possible due to the lack of a satisfactory in vitro system, although catecholamines certainly do have an effect in the intact animal (184). The mechanism of NST in skeletal muscle is still very obscure also because the changes observed upon cold acclimation are very low in amplitude compared with those occurring in BAT.

The plasma membrane (sarcolemma) is not dramatically altered upon cold adaptation (37). There is no apparent change in the polypeptide composition and in the lipid composition of the sarcolemma apart from a slight increase in the degree of unsaturation of fatty acids in the PS + PI fractions. There is a decrease in the binding of β-adrenergic ligands upon cold adaptation (37) or isoproterenol treatment (287). A high circulating level of catecholamines probably induces a desensitization of the adrenergic receptors. There is no change in the adenylate cyclase activity (37, 188) or in the 5'-nucleotidase and Mg^{++}-ATPase (37). An increase in the Na^+/K^+-ATPase is observed in C-A animals but no

effect of NE could be detected on the enzyme in vitro (37).

The mitochondria from skeletal muscles are altered in rats that are acclimated to cold. Their morphology is different. They become smaller and more numerous although their total mass is unchanged (14). This change in morphology is associated with the adaptive increase in the capacity to respond to NE, since both phenomena disappear together during deacclimation to cold and the appearance of both is inhibited when the rats are treated with oxytetracycline (an inhibitor of mitochondrial protein synthesis) during acclimation to cold (14). Their protein metabolism is also altered as indicated by a decreased half life of some groups of proteins in vivo (26) and a reduced incorporation of amino acids in vitro (27) although the polypeptide pattern of the mitochondrial membranes from C-A rats is not significantly different from that of W-A rats (132). The phospholipid composition of the mitochondrial membrane also appears slightly different (132). Isolated mitochondria from skeletal muscles of C-A rats incubated in presence of albumin are not, however, uncoupled or loosely-coupled as are the mitochondria from BAT. ADP/O ratios, Ca^{++}/O ratios and respiratory control with ADP or Ca^{++} are all normal (101, 130). There is an increase in state 3 respiration and a smaller increase in state 4 respiration with some substrates. The specific activities of some enzymes (glycerophosphate dehydrogenase, malate dehydrogenase and adenine nucleotide translocase) are changed (130, 132). However, isolated mitochondria from skeletal muscles of cold-acclimated fur seal pups are loosely-coupled in absence of albumin (98). These mitochondria show normal ADP/O ratios in presence of albumin but not in its absence while mitochondria from the warm-acclimated animals are tightly coupled in any

circumstances. The loose-coupling mechanism is different from the one observed in brown fat mitochondria since the addition of GTP has no effect on the respiratory control of these muscle mitochondria. Muscle mitochondria of the cold-acclimated fur seal pup have then a higher respiratory capacity and a control of respiration different in the cold-acclimated state from the warm-acclimated state and from the one occurring in the brown fat mitochondria. The nature of the loose-coupling of these mitochondria is not understood.

The mechanism of action of catecholamines to enhance the respiration of the skeletal muscle is still unknown. Several hypotheses have been proposed but the only one receiving some support is the possible acceleration of Ca^{++} -cycling that might occur in the muscle mitochondria of C-A rats. The rate of Ca^{++}-uptake, of Ca^{++}-stimulated respiration and of state 4 respiration after Ca^{++}-uptake are all increased in the altered mitochondria (101). The increase in rate of Ca^{++}-uptake also occurs gradually during the first 3-5 weeks of acclimation to cold. Na^{+} -induced Ca^{++}-release also occurs in these mitochondria but no difference is observed between W-A and C-A animals. Ca^{++}-cycling has also been postulated as a thermogenic mechanism in BAT (43, 135) and to explain the loose-coupling of the mitochondria from the abnormal skeletal muscle from people suffering from Luft's disease (a form of non-thyroidal hypermetabolism) (65). All these results have been obtained in vitro and there is at present no evidence for the occurrence of such Ca^{++}-cycling in vivo. In addition, if loose-coupling of the mitochondria through the acceleration of Ca^{++}-cycling is to be accepted as the thermogenic mechanism in skeletal muscle, there must be also a control mechanism under the influence of catecholamines which is altered upon cold adaptation. However, the physio-

logical mediator of the NE action has not yet been identified.

PART V. SUMMARY AND CONCLUSION

Brown adipose tissue is the major site of nonshivering thermogenesis, i.e. of the much enhanced calorigenic response to catecholamines. Skeletal muscle appears to be a minor contributor to the overall thermogenesis. In comparing the two tissues, one must not overlook the fundamental difference in their function in the animal. BAT is specialized for heat production while the main function of skeletal muscles is to allow movement via contraction. It is then obvious that, as far as heat production is concerned, the whole biochemical machinery of the brown fat may be devoted to this purpose while in muscle, controls must be superimposed so as not to impede its main function. But, a cell is a very efficient machine and controls exist in both tissues in order to switch on and off the energy dissipating process when needed. Evidence has been presented to indicate that different control mechanisms may contribute to a different extent during short-term and long-term cold exposure. On acute cold exposure, it appears that the control of the thermogenic activity is rapid, but the capacity is limited. Thus, the number of cells and mitochondria do not change and the control is achieved mainly by an energy dissipating reaction at the mitochondrial level in BAT. BAT mitochondria are indeed unique in possessing a specific and controllable proton conductance pathway, sensitive to purine nucleotides, that allows a high rate of respiration when open. The increase in heat production is mediated by NE directly released in the tissue from the abundant adrenergic innervation. On acute cold exposure, heat is produced in skeletal muscle from the hydrolysis of ATP for contraction (shivering). There is no adrenergic synapse on the skeletal muscle sarcolemma. Shivering is mediated via cholinergic innerva-

tion and the catecholamines reaching the tissue are coming from the circulatory system.

On the other hand, on long-term cold exposure, both BAT and skeletal muscle develop an enhanced capacity to respond to catecholamines. Figs. 10 and 11 depict the main hypotheses proposed to explain the mechanism of nonshivering thermogenesis in these two tissues. The physiological intracellular mediator of NE action is unknown in both cases. In BAT, there is growth of the tissue and an increase in its mitochondrial content. In skeletal muscle, there is an alteration of the mitochondrial morphology. The mitochondria of both tissues develop an enhanced capacity for futile ion cycling, i.e. H^+ in BAT and possibly Ca^{++} in muscle. The hormonal agent responsible for these changes is also unknown. In both cases, it is important to note that the mitochondria are said to be "loosely-coupled" so that some ATP production is still possible even under condition of active thermogenesis. This might be particularly important in skeletal muscle where certainly a tighter control may be needed to switch on and off the energy dissipating cycle when ATP is needed in larger amount. The basic idea of heat production via mitochondrial loose-coupling is then present in both tissues but in different form, most probably to take into account the different functions of the tissues.

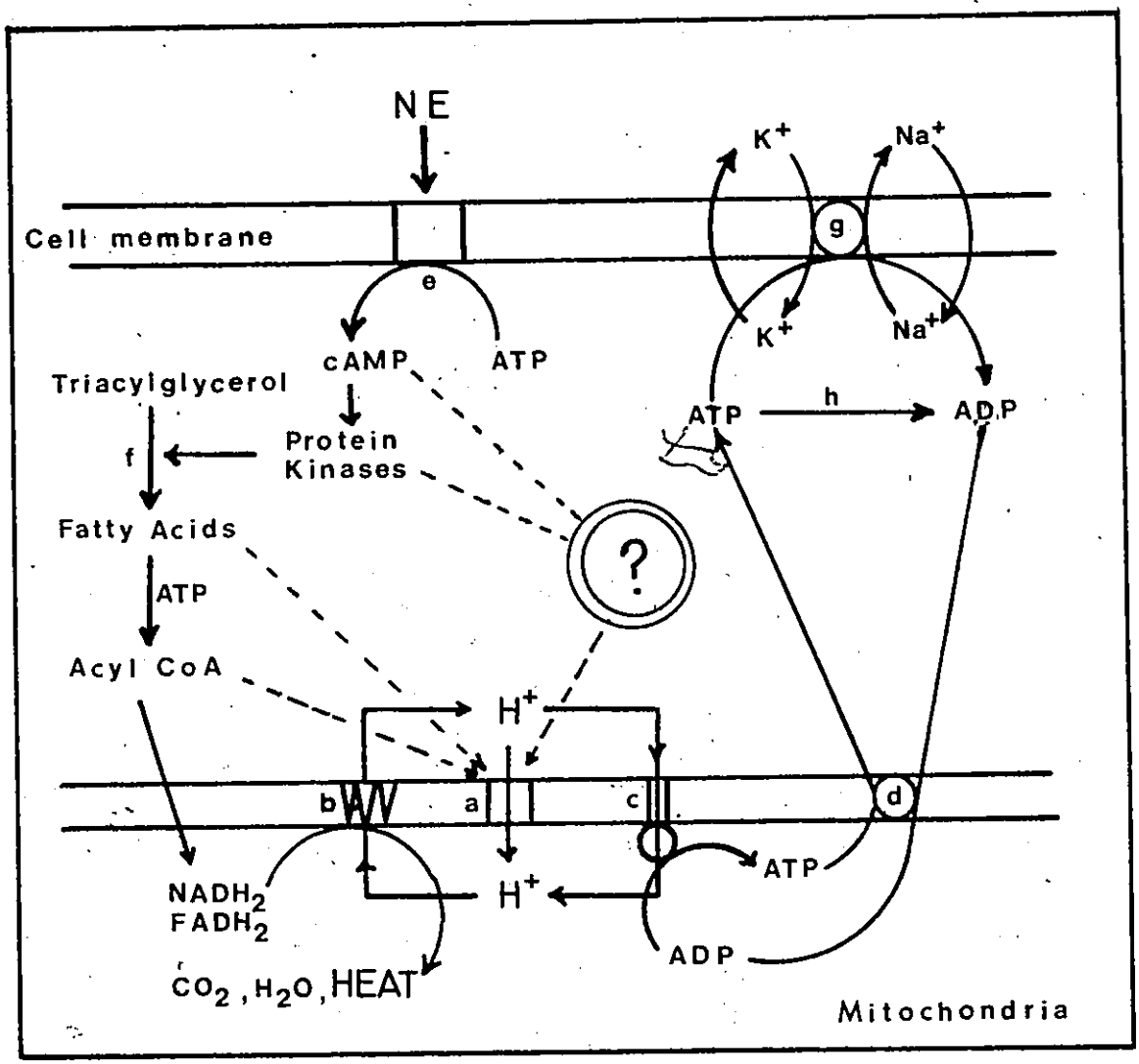

FIG. 10. Nonshivering thermogenesis in brown adipose tissue.

The broken lines represent the possible origins of the message increasing the effective proton conductance of the mitochondrial nucleotide-sensitive conductance pathway (a); (b) is the respiratory chain; (c) the ATP-synthetase; (d) the adenine nucleotide translocase; (e) the adenylate cyclase; (f) the hormone sensitive lipases; (g) the Na^+/K^+-ATPase; (h) other ATPases.

The increased thermogenesis in this tissue results from the enhancement of mitochondrial proton cycling (circuit (b) -- (a)).

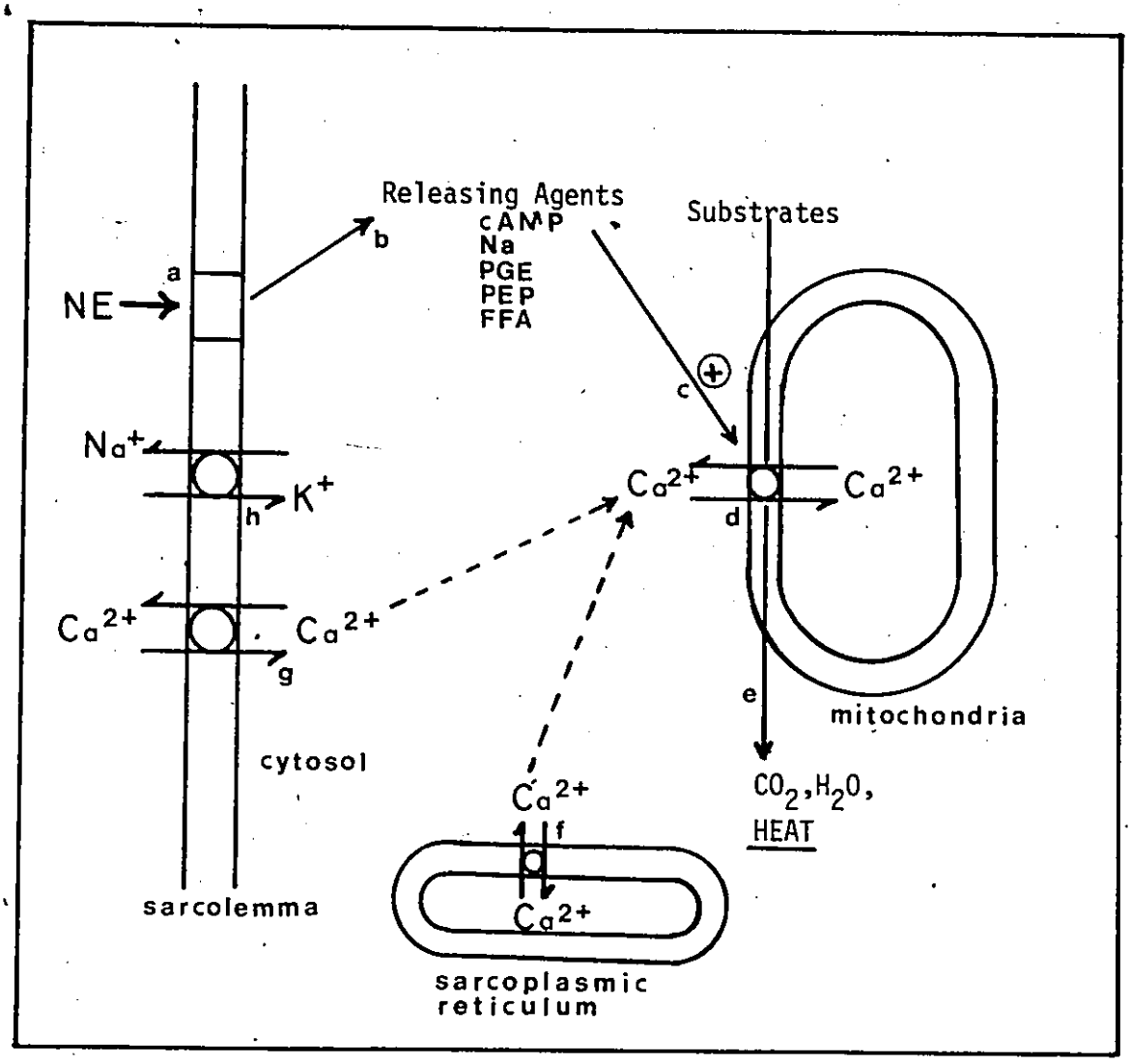

FIG. 11. Nonshivering thermogenesis in skeletal muscle.

NE acts upon its receptor in the sarcolemma (a), causing an increase in the cytosolic concentration of a releasing agent (b), which in turn enhances calcium efflux from the mitochondria (c). This makes more calcium available for energy-linked re-uptake (d), and respiration is stimulated (e). Other potential sources of calcium are the sarcoplasmic reticulum (f) and the extracellular fluid (g). Also shown is the Na^+/K^+ ATPase (h) which may be involved indirectly in calcium movements. (From Greenway, 100).

STATEMENT OF THE PROBLEM

The original objective of the work described in this thesis was to define the importance of the proton conductance pathway of BAT mitochondria in heat production by the tissue. To this end, BAT mitochondria from cold-acclimated rats (known to have a very large capacity for NE-induced thermogenesis in their BAT) were compared with mitochondria from warm-acclimated rats (known to have a much smaller capacity for NE-induced thermogenesis in their BAT than C-A rats). It was predicted that any differences would be related to the different capacity for thermogenesis. When the work was started (1976-77) it had just been reported that the proportion of a 32 000 polypeptide was increased in BAT mitochondria of C-A rats (Ricquier and Kader, 1976; Himms-Hagen et al., at the Conference on Effectors of Thermogenesis, 1977) and Nicholls had just reported (Conference on Effectors of Thermogenesis, 1977) that purine nucleotides, inhibitors of the proton conductance pathway, bind to a polypeptide of molecular weight 32 000 in hamster BAT mitochondria. Initial experiments therefore concentrated on a comparison of polypeptide composition and purine nucleotide binding as indirect indicators of the proton conductance pathway. Changes observed were correlated with some functional characteristics (proton conductance, respiration) and the ultrastructure (electron microscopy) of the mitochondria.

Once marked differences in composition and properties had been established, the next objective was to find out whether the changes were acute and rapid responses to the stimulus of exposure to cold or whether they were slower adaptive changes related to the slowly developing increased capacity for thermogenesis. In order to distinguish between

these two possibilities the time-course of appearance and disappearance of the changes during acclimation to cold and during deacclimation were studied.

The final objective was to find out how the observed changes were induced during acclimation to cold. The role of protein synthesis in bringing about the altered composition of the mitochondria was studied by the use of protein synthesis inhibitors. The nature of the mediator of the cold-induced changes in the intact animal was studied by long-term treatment of animals with potential mediators such as norepinephrine and thyroid hormone. The nature of the intracellular mediator was studied by attempting to bring about similar changes in isolated mitochondria.

During the period in which this work was performed (1976-1980) several other research laboratories, working independently, reported results which confirm or complement many of those reported here. For example, although it was known in 1976 that BAT mitochondria of hamsters (Nicholls, 196) and newborn guinea pigs (Rafael and Heldt, 225) have a large capacity to bind purine nucleotides, only in 1978 did we (Desautels and Himms-Hagen, 64) and Cannon (Cannon et al, 33) report the enhanced binding in BAT mitochondria of the cold-acclimated rat. Papers demonstrating the major role of BAT in nonshivering thermogenesis appeared only in 1978 (Foster, 84, 85) and 1979 (Foster, 86). The full report that the 32 000 polypeptide is the binding site for the purine nucleotides in hamster BAT mitochondria appeared only in 1978 (Heaton et al, 114) and was confirmed in 1979 (Ricquier, 235). Discussion of the results presented in this thesis will include a comparison of the related findings from these other research groups, reported simultaneously or after the present work was done.

The ultimate goal of the research described in this thesis is the description in biochemical terms of the nature and control of nonshivering thermogenesis. Such knowledge may be particularly important at a time when recent biomedical research points to BAT as a regulator of overall energy balance, not only in relation to cold but also in relation to diet, and not only in small mammals but possibly also in man.

MATERIALS AND METHODS

PART I. MATERIALS

A. Rats

Male Charles River Holtzmann rats were obtained (Canadian Breeding Laboratories) at an initial weight of 100-125 g. After at least a week in group cages (about 8-10 rats/cage) at 28°C, they were transferred to individual metal cages with free access to food and water and with 12 hours of artificial lighting a day (6:00 - 18:00 hrs). At the time of the transfer, the rats weighed 170-250 g. They were then maintained at either 28°C (referred to as warm-exposed) or 4°C (referred to as cold-exposed) for the times indicated. Cold- or warm-acclimated rats were kept in their respective temperature environment for a minimum of 4 weeks before use. The animals were weighed weekly and just before the experiments; only rats showing normal growth were used. The experiments were designed so that the animals were studied in pairs with only one factor varying at the time in order to reduce the variability between the rats due to age, sex, duration of exposure to cold, age of initial exposure to cold, diet, handling and to reduce the variability in experimental procedures due to the equipment, the solutions and the manipulations. In some experiments, it was necessary to pool the tissues from several animals in order to obtain enough material.

B. Chemicals

Biochemicals of the finest purity commercially available were purchased from Sigma Chemical Co. Common laboratory reagents such as NaOH, KCl, were obtained from Fisher Scientific Co. or J.T. Baker Co.

Chemicals and apparatus needed for gel electrophoresis were purchased from Bio-Rad Laboratories. The electron microscopy technique is very sensitive to the source and the state of the chemicals used. A list of the compounds used as well as their source is then needed here. Absolute ethyl alcohol was obtained from the Ontario Liquor Control Board; styrene from Eastman Kodak Co.; osmium tetroxide from Electron Microscopy Sciences; glutaraldehyde 70% from Ladd Research Laboratories and Vestopal W from Mrs Martin Jaeger, 1222 Vésenaz, Geneva, Switzerland.

All radioactive compounds were purchased from New-England Nuclear; {U-^{14}C} Sucrose, 3.6-4.9 mCi/mmole; {2,8-^{3}H} adenosine 5'-diphosphate, trisodium salt, 24.8-29.1 Ci/mmole; {8-^{3}H} guanosine 5'-diphosphate, trisodium salt, 8.04-8.91 Ci/mmole; L-{3,4,5-^{3}H(N)} leucine, 118.4 Ci/mmole; {^{14}C} methylamine hydrochloride, 50.1 mCi/mmole; {^{3}H} acetic acid, sodium salt, 150.0 mCi/mmole; {^{86}Rb} rubidium chloride, 240 mCi/mmole. The NCS tissue solubilizer and PCS (liquid scintillation counting cocktail) were obtained from Amersham Corporation.

PART II. METHODS (General)

A. Isolation of brown adipose tissue mitochondria

Shortly before the experiment, rats were taken to the laboratory from their respective temperature-controlled rooms, weighed and killed by decapitation. The interscapular brown adipose tissue was removed, cleaned of adhering white fat and muscle, and weighed. The tissue was then minced with scissors, and homogenized in cold isolation medium containing 0.25 M sucrose, 0.2 mM EDTA (disodium salt) and 1 mM HEPES (pH 7.2). The mitochondria were then isolated exactly as described by Slinde, Pedersen and Flatmark (263). The centrifugations were performed in a Sorvall RC-2B refrigerated centrifuge, using the HB-4 rotor. The brake of the centrifuge was turned off. The final mitochondrial pellet was resuspended in the isolation medium at a concentration of 4-10 mg/ml and kept on ice until use. Mitochondria were used as soon as possible, no longer than 4 hours after the end of the isolation procedure: The purity of the mitochondrial preparation was routinely checked by phase-contrast microscopy with a Carl-Zeiss photomicroscope (magnification x 640).

B. Protein estimation

Protein was determined by the Lowry method (180), as modified by Schacterle and Pollack (245). Bovine serum albumin was used as standard. Because a number of substances, including sucrose, tris, EDTA and HEPES have been found to interfere with the Lowry protein determination (158), homogenate or mitochondrial samples used for protein estimation were precipitated with cold 12.5% trichloroacetic acid, left to stand on ice for 30 minutes and centrifuged at 16 500 x g for 15 minutes. The pellets

obtained were dissolved in 2.0 ml of 0.5 N NaOH and 1.0 ml of this solution assayed (245).

C. Cytochrome oxidase activity assay

Samples of BAT homogenates (see II-A) were activated with Lubrol (3 mg/mg protein) and kept frozen until use. The activation was performed by re-homogenizing the samples in the presence of Lubrol for one minute in 1-5 ml Teflon-glass homogenizer at 10 000 r.p.m. The sample volume, its dilution ratio, the duration of the homogenization as well as the number of r.p.m. of the pestle are important for the reproducibility of the results. Cytochrome oxidase activity was measured polarographically at 37°C in a medium containing 0.1 M potassium phosphate buffer pH 6.6, 0.2 mM cytochrome c and 20 mM sodium ascorbate in a volume of 3.0 ml as described previously (14). The cytochrome c solution was prepared by dissolving 1 g of cytochrome c in 100 ml of 0.01 M potassium phosphate buffer pH 7.4 containing 100 mg of ascorbic acid as described by Wharton and Tzagoloff (290). The concentration of the total and reduced cytochrome c was checked using the extinction coefficients reported by Yonetani (294). The activity is expressed as µatoms oxygen consumed /min·mg protein. The total cytochrome oxidase activity of a given tissue is obtained by multiplying the specific activity by the total amount of protein in the tissue studied.

D. Purine nucleotide binding measurement

Purine nucleotide binding sites were assessed from the binding of ^{3}H-labelled GDP or ^{3}H-labelled ADP in the presence of atractyloside, essentially as described by Nicholls (196). Mitochondria (final concentration 0.2-0.6 mg/ml) were added to 0.75 ml (final volume) of incubation medium

at pH 7.1 and room temperature containing 100 mM sucrose, 20 mM N-Tris (hydroxymethyl)-2-aminoethane sulphonate, 10 mM choline chloride, 1 mM EDTA disodium salt, 5 µM rotenone, 100 µM potassium atractyloside and 0.1 µCi/ml of ^{14}C-sucrose. After 1 minute, the reaction was started by the addition of either ^{3}H-labelled ADP or ^{3}H-labelled GDP (1.25 µCi, final concentration 10 µM). Mixing was rapidly done with a Vortex mixer. After 30 sec., the mitochondria were centrifuged down rapidly (2 min., Eppendorf microcentrifuge) and the supernatant was removed with the help of a Pasteur pipette attached to a water pump. The mitochondrial pellet was then dissolved by incubation in NCS tissue solubilizer overnight at 55°C, and counted (liquid scintillation counting in the presence of 0.05 ml of 10% ascorbic acid and 7.0 ml toluene containing 0.7% w/v PPO). Binding of the nucleotides was calculated from the amount of tritiated nucleotide present after correction (from the amount of ^{14}C-sucrose present) for the amount trapped in the water in the pellet. Parallel samples containing 100 µM unlabelled ADP or GDP used to displace the bound ^{3}H-labelled GDP or ^{3}H-labelled ADP respectively were run in order to estimate the amount of unspecific binding. The amount of bound ^{3}H-GDP displaced by the unlabelled ADP has been taken as a measure of the specific binding of GDP. Similarly, the amount of bound ^{3}H-ADP displaced by the unlabelled GDP corresponds to the specific binding of ADP. The results are expressed as nmoles GDP (or ADP) bound / mg mitochondrial protein.

E. Polypeptide composition

SDS-polyacrylamide gel electrophoresis were performed according to Weber et al (289). Mitochondria at a protein concentration of 2 mg/ml were sonicated with the microprobe of the Bronwill Biosonik III at the maximum intensity for 1.5 minute. The sample was kept on ice and sub-

jected to 3 short sonications of 0.5 minute each. After a 60 minute centrifugation at 40 000 x g, the sedimented membranes were dissolved in solvent buffer containing 10 µM phenylmethysulfonylfluoride, heated at 100°C for 2 minutes, and subsequently, electrophoresed on 6% polyacrylamide gels in 0.1 M sodium phosphate buffer pH 7.2 with 0.1% SDS and 10 µM phenylmethylsulfonylfluoride. Gels were stained with Coomassie blue R250, destained by diffusion, and scanned at 540 nm with the Gilford spectrophotometer 2400-2. The proportion of the total area under each peak (expressed as %) was assessed by cutting out 2 copies of each tracing and weighing each major peak.

F. Mitochondrial size distribution

The size distribution of brown fat mitochondrial suspensions was estimated with a Coulter Counter (model ZB) equipped with a Channelyzer and a 10 micron aperture tube. The determination of the size distribution depends on the particle volume and on the difference in electrical resistance between the electrolyte solution and the particles suspended in it. A more detailed description of the theory and practice of the method is given in appendix A.

The mitochondrial suspension was diluted 4000-10000 x (1-10 µl/20 ml) in an ionic medium containing 0.25 M sucrose, 0.06 M potassium chloride, and 0.02 M N-Tris (hydroxymethyl)-2-aminoethane sulfonate at pH 7.2 and room temperature to yield a final protein concentration of 0.3-1.1 µg/ml. The diluent solution had been previously thoroughly filtered (twice with 0.8 µ Millipore filter followed by three 0.22 µ filtrations) to remove any dust or non-mitochondrial particles. The particle counts for each of 100 separate windows (threshold divisions) were then recorded. The counts obtained for the medium alone ("blank") were subtracted. Because

of frequent blockages of the very small aperture used, the counting time was kept very short and multiple counts were taken (four for each of two different dilutions of the same mitochondrial suspension) and averaged. The particle number at each threshold division (count - blank) was then corrected to a protein concentration of 1 µg/ml and multiplied by the volume of the particle at that division to obtain the total mitochondrial volume contributed by mitochondria of that size. The calibration factor was 0.02181 μm^3/threshold division determined as described previously (90), using standard latex particles of 0.5 µm and 1.099 µm diameter. The counter was allowed to warm up several hours before use with the following settings: 1/aperture current = 4; 1/amplification = 2; matching switch = 160 K; gain = 3.5 and time for counting = 10 seconds. The volume actually flowing through the aperture in 10 seconds was estimated to be 3.147 ± 0.047 µl (n = 5). The results are expressed as the total volume in μm^3 contributed by mitochondria of sizes ranging from 0.0218 μm^3 to 2.181 μm^3.

G. Electron microscopy of BAT mitochondria

The BAT mitochondria were isolated as in II-A except that the last resuspension-centrifugation step was done in EDTA-free isolation medium (sucrose 0.25 M; HEPES 1 mM; pH 7.2). The mitochondria were also finally resuspended in EDTA-free isolation medium. The fixation was performed according to Munn and Blair (190), adding 0.8 ml of the mitochondrial suspension at a protein concentration of 2-6 mg/ml to 6.7 ml of fixative solution containing 2.5 ml of 6% glutaraldehyde in 0.02 M sodium cacodylate buffer pH 7.0 and 4.2 ml of EDTA-free isolation medium. The final concentration of glutaraldehyde was then 2%. This has been shown to stop immediately any swelling or contraction of the mitochondria as determined by changes in extinction at 520 nm (190). The mitochondrial suspension was kept on ice for 1 hour and subsequently centrifuged for

1 hour in the HB-4 rotor of a Sorvall RC-2B refrigerated centrifuge at 4100 x g. The supernatant was then replaced by 3.0 ml of 1% osmium tetroxide in 0.02 M sodium cacodylate buffer pH 7.0. The pellet was broken into small fragments with a finely drawn glass rod and kept at room temperature. After one hour, the osmium tetroxide solution was replaced and the fragments allowed to remain in the osmium tetroxide for another hour. The fragments were dehydrated successively in 50%, 75%, 95% and 100% ethanol, transferred to styrene and embedded in Vestopal W. The actual procedure for the dehydration of the samples and their embedding in Vestopal W is given in appendix B. The impregnation with Vestopal was allowed to proceed for 2 days at room temperature as recommended by Nunn (207) before polymerization at 60°C for 4 days.

Thin sections were cut with a Reichert Ultramicrotome UM-2 using glass knives. Sections with silver to gold interference colors were mounted on formvar-carbon coated 200 mesh copper grids. The sections were stained with uranyl acetate (saturated solution in 60% ethanol) for 10 minutes followed by lead citrate (233) for another 10 minutes. The grids with sections are stained by floating upside down on single drops of staining solutions. Following staining, the grids are washed in distilled water and allowed to dry. The specimens were then viewed in a Siemens 101 electron microscope at 8000 x and 12000 x magnification.

H. Statistical analysis of the results

Results are expressed as means ± standard error of the mean (SEM). The significance of the differences observed was calculated using the unpaired t-student test.

$$t = \frac{M_1 - M_2}{\sqrt{\varepsilon_1^2 + \varepsilon_2^2}} \quad \text{with } n_1 + n_2 - 2 \text{ degrees of freedom}$$

where M_1 and M_2 are the means, ε_1 and ε_2 the standard errors, n_1 and n_2 the number of observations of the two experimental groups under comparison.

RESULTS AND DISCUSSION

PART I. DIFFERENCES BETWEEN BROWN ADIPOSE TISSUE MITOCHONDRIA OF COLD-ACCLIMATED RATS AND BROWN ADIPOSE TISSUE MITOCHONDRIA OF WARM-ACCLIMATED RATS

A. DIFFERENCES IN COMPOSITION BETWEEN BAT MITOCHONDRIA OF C-A AND W-A RATS

BAT mitochondria are unique in possessing a specific purine nucleotide sensitive proton conductance pathway (195, 196). This pathway is believed to be responsible for futile proton cycling across the inner membranes resulting in a de-energization of the mitochondria and a subsequent enhancement of their respiratory rates (195, 200). Purine nucleotides are known to inhibit futile proton cycling in hamster BAT mitochondria (200). It has also been demonstrated that in hamster or newborn guinea pig, purine nucleotides bind to a specific site different from the adenine nucleotide translocase, on the outer surface of brown fat mitochondrial inner membrane (196, 225). In addition, the extent of binding appears related to the thermogenic state of the tissue. Binding of purine nucleotides to brown fat mitochondria isolated from hamster or newborn guinea pigs is much higher than binding to mitochondria isolated from fetal or adult guinea pigs in which BAT thermogenesis is known to be minimal (196, 225). Similarly, the binding site is not apparent in rat liver mitochondria, a non-thermogenic tissue (196). In 1976-77, an increase in a 32 000 polypeptide was reported in BAT mitochondrial membrane from cold-acclimated rats (132, 236). Independently, this polypeptide was identified as being the binding site of the purine nucleotides in hamster BAT mitochondria (202).

Objective: The initial objective of this work was to compare purine nucleotide binding and the proportion of 32 000 polypeptide in BAT mitochondria of cold- and warm-acclimated rats.

1. Purine nucleotide binding to isolated BAT mitochondria of cold- and warm-acclimated rats

The concentration of binding sites as well as their affinity toward purine nucleotides were studied in isolated brown fat and skeletal muscle mitochondria from warm- and cold-acclimated rats.

Method: The purine nucleotide binding was assessed from the binding of ^{3}H-labelled GDP and ^{3}H-labelled ADP in presence of atractyloside as described in the method section with the following slight modifications. The mitochondria were added to stirred incubation medium (final volume 3.0 ml) in presence or absence of a competing amount (100 µM) of unlabelled ADP or GDP to displace the bound ^{3}H-ADP respectively. 0.5, 3 and 6 minutes after the start of the reaction (addition of the tritiated nucleotides), samples (1.0 ml) were removed and the mitochondria separated by centrifugation and treated as described previously (Methods II-D)

Twelve warm-acclimated rats (314 ± 14 g) and 4 cold-acclimated rats (226 ± 18 g, acclimated for 3-5 weeks) were used for the isolation of the mitochondria and measurement of the purine nucleotide binding.

For comparison, mitochondria from mixed leg muscles were isolated from 6 W-A rats (365 ± 12 g) and 6 C-A rats (316 ± 13 g, acclimated for 6-8 weeks) as described by Behrens and Himms-Hagen (14). Purine nucleotide

binding was assessed as described above.

Scatchard plot analysis of GDP binding to BAT mitochondria was also performed. For this purpose, 17 W-A rats (501 ± 14 g) and 6 C-A rats (465 ± 23 g, acclimated for 8-12 weeks) were used. The GDP binding was measured exactly as described in the methods section (II-D) except that the final GDP concentration was varied from 0.5-50 μM. The concentration of unlabelled ADP to displace the bound ^{3}H-GDP was raised to 1 mM.

Results and discussion: The binding of both GDP and ADP is greatly increased in BAT mitochondria of rats acclimated to cold for 3-5 weeks (Fig. 12). The binding of ^{3}H-labelled GDP was reduced by an excess of ADP and the binding of ^{3}H-labelled ADP was reduced by an excess of GDP. This confirms the competitive nature of purine nucleotide binding to the proton conductance sites as originally described by Nicholls (196). Because of this unique characteristic of the proton conductance pathway, the amount of bound labelled nucleotide displaced by the other unlabelled nucleotide at 0.5 minute has been taken as a specific measure of the number of binding sites. The specific binding of GDP to BAT mitochondria is 0.41 ± 0.04 nmoles/mg protein for the C-A rats and 0.04 ± 0.01 nmoles/mg protein for the W-A rats ($p < 0.001$). The specific binding of ADP was always slightly lower, most probably because of the lower affinity of the binding sites for ADP (113). The specific binding of ADP is 0.31 ± 0.02 nmoles/mg protein for the C-A rats and 0.03 ± 0.00 nmoles/mg protein for the W-A rats ($p < 0.001$). The non-specific binding of GDP (that part not eliminated by ADP) but not of ADP (that part of ADP binding not eliminated by GDP) was higher in the C-A rats. The meaning of this

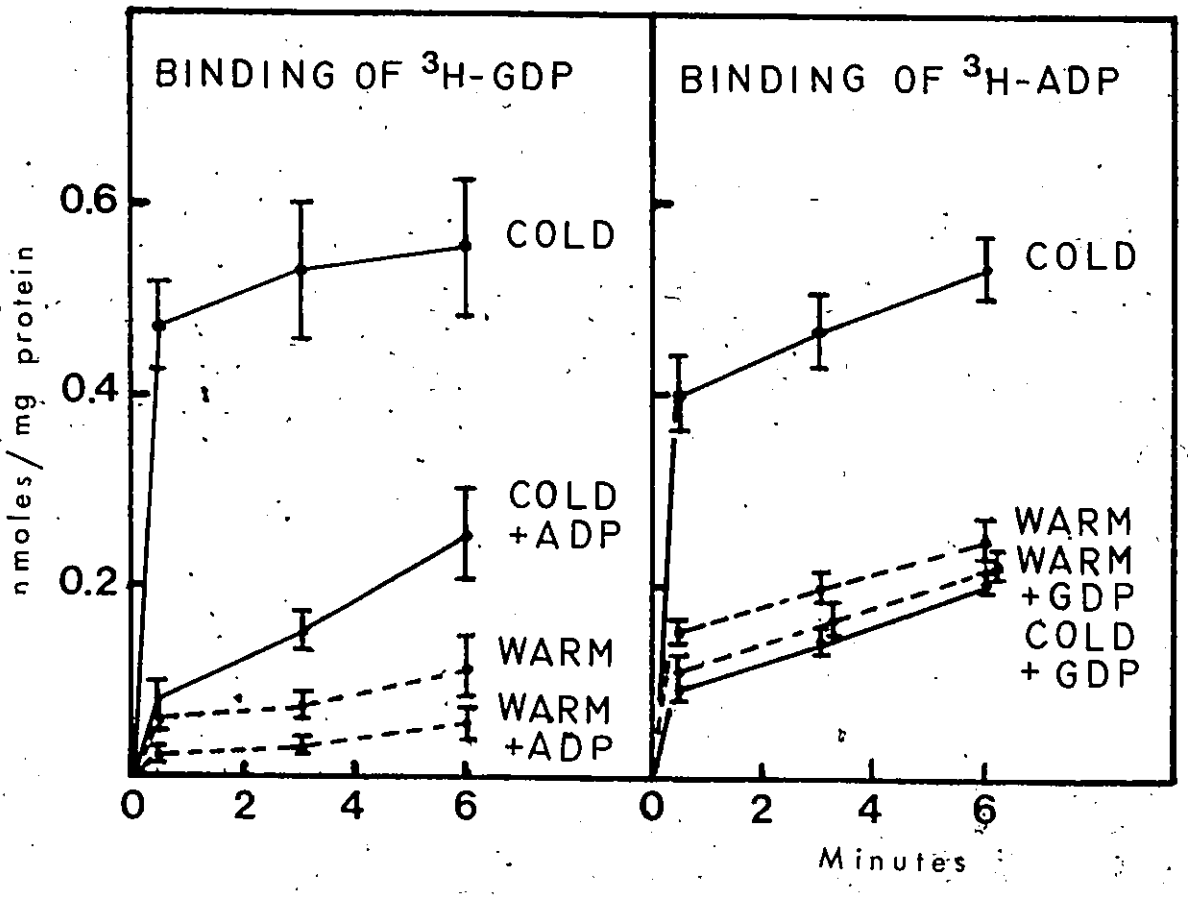

FIG. 12. Time course of association of ^{3}H-labelled GDP (10 µM, 1.25 µCi) and of ^{3}H-labelled ADP (10 µM, 1.25 µCi) with atractyloside pre-treated brown adipose tissue mitochondria of cold-acclimated (COLD) or warm-acclimated (WARM) rats. The concentration of unlabelled ADP or GDP used to displace the bound ^{3}H-GDP or ^{3}H-ADP was 100 µM. Values are the means (±S.E.) of 4 observations.

observation is not clear. The binding values obtained in this work for the C-A rats compare well with already published data on BAT mitochondria of hamster (196) or newborn guinea pig (225). The binding values for the W-A rats (28°C) are lower than those reported for 20 days old guinea pigs kept at 22°C (225). Since GDP is not translocated into the mitochondria under any circumstances, it is a more reliable marker than any other purine nucleotides for the determination of the concentration and affinity of the proton conductance pathways. The binding affinity of those sites for GDP has been estimated. At pH 7.1, Scatchard plot of GDP binding to BAT mitochondria of warm- and cold-acclimated rats confirms the increased concentration of a single class of binding sites in C-A animals (Fig. 13). There is no difference in binding affinity toward GDP between the two mitochondrial preparations as judged from the similarity of the slopes in Fig. 13. The actual GDP affinity constants have been obtained from linear regression analysis of the binding data. At pH 7.1, they are 6.33 μM and 6.49 μM for cold- and warm-acclimated rats respectively. For comparison, Nicholls reported a GDP affinity constant of 4.2 μM at pH 6.7 and increasing to 30 μM at pH 7.9 in hamster BAT mitochondria (196).

In contrast, the specific purine nucleotide binding to skeletal muscle mitochondria is very low and is not modified by adaptation to cold. The number of ^{3}H-GDP binding sites are 0.011 ± 0.004 nmoles/mg protein and 0.010 ± 0.003 nmoles/mg protein for the warm- and cold-acclimated rats respectively (p = N.S.) These results are consistent with the recently reported lack of response after addition of GTP of the respiratory rates of mitochondria from muscles of warm- and cold-acclimated fur seal pups (195).

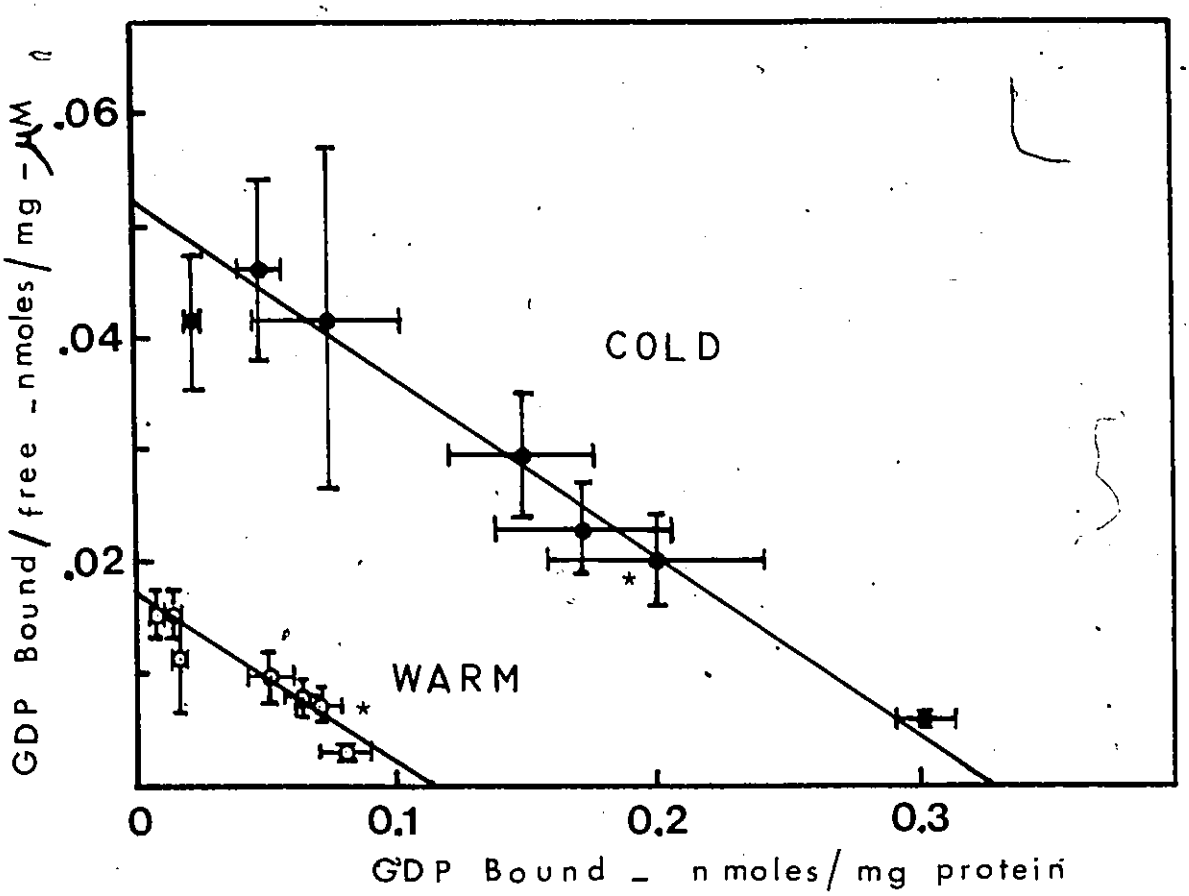

FIG. 13. Scatchard plot of binding of GDP by BAT mitochondria of warm-acclimated (WARM) or cold-acclimated (COLD) rats. Values are the means (±S.E.) of 5 observations. The intercept on the x-axis gives the number of sites present while the slope of the line gives the affinity constant.

The two points with the (*) correspond to the usual conditions for measuring purine nucleotide binding by BAT mitochondria from W-A and C-A rats.

2. Polypeptide composition of BAT mitochondrial membranes of cold- and warm-acclimated rats

Method: The polypeptide composition of mitochondrial membranes was obtained by polyacrylamide gel electrophoresis exactly as described earlier (Method II-E). Two W-A and two C-A rats (6 weeks at either 28°C or 4°C respectively) were used for the experiment.

Results and discussion: The polypeptide composition of the BAT mitochondrial membranes of W-A and C-A rats is illustrated in Fig. 14. The major differences between warm- and cold-acclimated rats observed consistently are an increase in a polypeptide of 32 000 M.W. (peak 14) and decreases in polypeptides of 96 000 M.W. (peak 6) and 50 000 M.W. (peak 12). The increase in the 32 000 M.W. polypeptide has been reported previously in the literature (236) and very recently, this polypeptide has been partially purified by GDP-agarose affinity chromatography (235). Interestingly, four polypeptides are eluted from the affinity chromatography column: two major polypeptides of 32 000 M.W. and 103 000 M.W. and two minor ones having molecular weights of 50 000 and 58 000. It is not known whether there is any relationship between these GDP-binding polypeptides and those reported in this thesis to decrease in cold-acclimated rats. The 50 000 M.W. polypeptide may be a subunit of the F_1-ATPase which is known to have polypeptides in the range 50 000 - 55 000 M.W. (36, 151). The relative proportion of F_1-ATPase is known to be lower in BAT than in non-thermogenic tissues (151) but it is not known whether it is also decreased upon cold adaptation. The polypeptide composition of skeletal muscle mitochondrial membranes of cold- and warm-acclimated

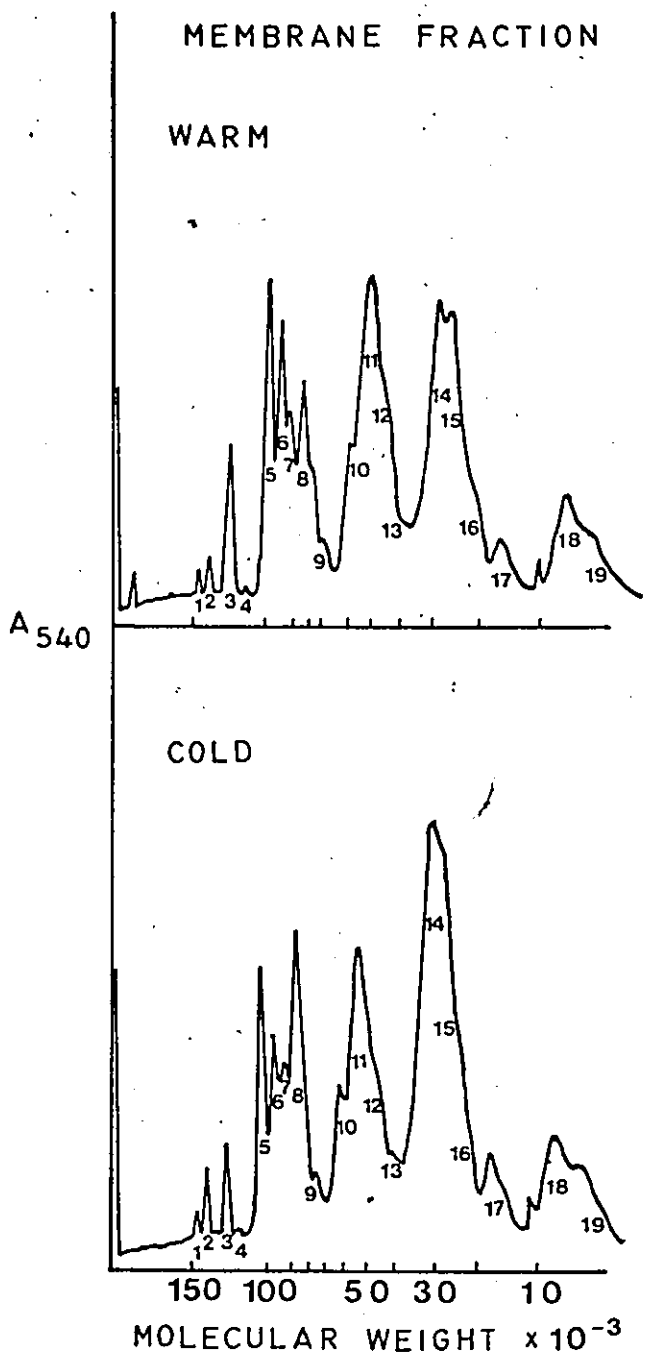

FIG. 14. Polypeptide pattern obtained by SDS-polyacrylamide gel electrophoresis of the membranes of brown adipose tissue mitochondria.

Upper section, mitochondria from rats acclimated to 28°C for 6 weeks (WARM); lower section, mitochondria from rats acclimated to 4°C for 6 weeks (COLD).

rats has also been reported in the literature (132). The polypeptide composition is very different from that one of BAT mitochondria and only minor changes are apparent after cold acclimation.

Conclusions: Both purine nucleotide binding and the proportion of a polypeptide of 32 000 M.W. (binding site of the purine nucleotides) are increased in BAT mitochondria of cold-acclimated rats. There is however no difference in binding affinity between mitochondria of W-A and C-A rats. An increase in the concentration of binding sites, and hence, of proton conductance pathways, is then responsible for the increase in the binding of purine nucleotides to mitochondrial membranes of cold-acclimated rats.

On the other hand, the very low binding capacity of isolated skeletal muscle mitochondria and the fact that it does not increase upon cold acclimation lead to the conclusion that there is a different mechanism of control of thermogenesis in this tissue.

B. DIFFERENCES IN STRUCTURE BETWEEN BAT MITOCHONDRIA OF C-A AND W-A RATS

Purine nucleotide binding and the proportion of a polypeptide of 32 000 M.W. are increased in BAT mitochondria of C-A rats. Since all the work presented in this thesis is performed on isolated mitochondria, the state of purity and structural integrity of the mitochondrial preparation is an important factor to consider, particularly when evaluating physical parameters such as binding of labelled nucleotides to mitochondrial membranes.

Objective: Ultrastructural studies of mitochondrial suspensions from W-A and C-A rats were performed in order to verify the purity of the preparations and to determine if any structural differences could not also partly account for the difference in binding of purine nucleotides to brown fat mitochondria. These ultrastructural studies include electron microscopy and measurement of size distribution of the mitochondrial suspensions.

1. Electron microscopy of isolated BAT mitochondria from W-A and C-A rats

Method: Four W-A rats and two C-A rats weighing respectively 355 ± 22 g and 353 ± 7 g were used. They were acclimated at either 28°C or 4°C for four weeks. The mitochondria were isolated and electron microscopy performed as described previously (Method, section F). Sections from several different regions of the mitochondrial pellet were viewed to ensure that the appearance was truly representative of the mitochondria isolated.

Results and discussion: Electron micrographs of BAT mitochondria from warm- and cold-acclimated rats are shown in Fig. 15 and 16. Mitochondria are recognized morphologically by the presence of two membranes, of which the inner one shows infoldings or cristae. Both mitochondrial preparations from W-A or C-A rats are characterized by an electron-dense matrix (black) and an electron transparent intracristal space (intermembrane space). Both preparations appear relatively free of contamination and have an excellent degree of preservation. Only a few mitochondria

FIG. 15. Brown adipose tissue mitochondria isolated from rats acclimated at 28°C for 4 weeks.
(x 16 000; Bar = µm)

FIG. 16. Brown adipose tissue mitochondria isolated from rats acclimated at 4°C for 4 weeks.
('x 16 000; Bar = µm)

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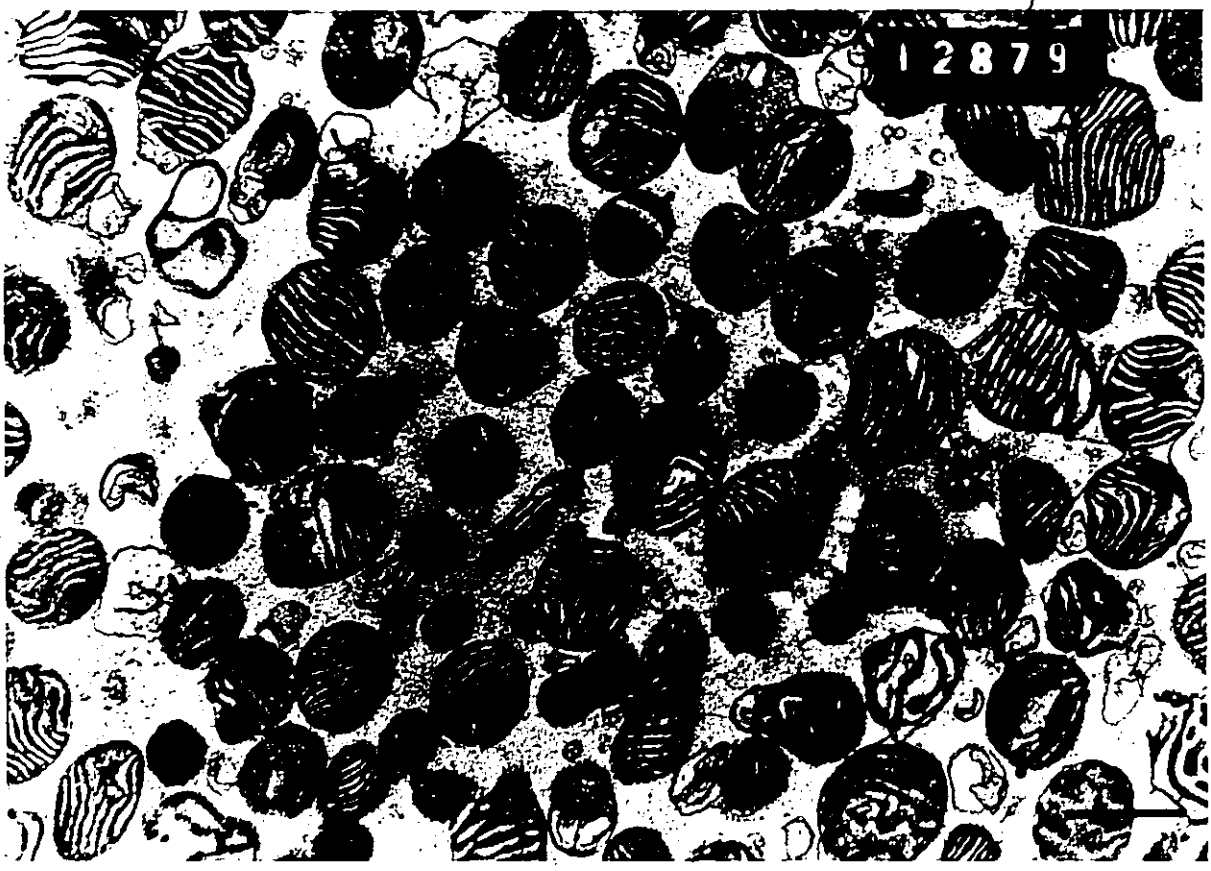

appear broken and the different regions of the mitochondria (outer and inner membranes, matrix and intracristal space) can easily be distinguished.

BAT mitochondria from C-A rats are characterized by numerous parallel cristae, which fill the whole of the transverse section of the organelle. The matrix appears condensed. In contrast, the electron micrographs of mitochondria from the W-A rats however, show a much less regular internal structure. The mitochondria have a large intracristal space and a very condensed matrix which give them a "dotted" appearance. The complete absence of the "dotted" mitochondria is to be remarked on the electron micrograph of the C-A rats. The appearance of the mitochondria from the W-A rats probably results from a lower amount of membrane material and/or a greater condensation of the matrix.

When compared with mitochondria observed in the tissue itself (270, 271, 279), the fine structure of sucrose-isolated mitochondria is obviously different from their appearance in situ. The ultrastructure of the isolated mitochondria presented in this thesis has been compared with that of mitochondria present in the tissue as reported in the literature. The difference is most evident for the W-A rats where the mitochondria in the BAT appear with cristae often curved and arranged in more or less parallel systems in an expanded matrix. In no case do the mitochondria ever appear with a "dotted" internal structure. The morphology of the mitochondria in the BAT of C-A rats does not however differ significantly from that one of the isolated mitochondria. They have numerous, closely spaced, straight cristae.

One problem in studying the mitochondrial structure by electron microscopy is the possible formation of artefacts by the fixation procedure.

However, the fixation of mitochondria in suspension as used in this study has been shown to stop immediately any swelling or contraction of the mitochondria and to result in consistent mitochondrial conformation throughout the entire depth of the pellet (104, 190). This technique actually avoids the conformational changes induced by anaerobiosis of the "old" technique where the fixation was performed during the centrifugation itself. Indeed, in this study, on several occasions, many sections from different blocks prepared from different regions of the same mitochondrial pellet were obtained and no difference was ever observed between the electron micrographs. However, the formation of artefacts from a difference in reactivity of the fixative to different mitochondria (C-A / W-A) or to mitochondria in different functional state can not be excluded.

In the literature, the appearance of sucrose-isolated BAT mitochondria from cold-adapted hamster (28), ground squirrels (280), newborn rabbits (179) and newborn (226) or cold-acclimated rats (226, 272) is very similar to that of the mitochondria from the cold-acclimated rats reported in this thesis. An early report about W-A rats that had been starved for several days shows mitochondria with an intermediate type of internal structure (in-between the "dotted" and the parallel arrangement of the cristae) (178). Interestingly, the isolated mitochondria from the BAT of the W-A rats look very similar to those isolated from white fat, although the matrix of the latter is in a less condensed form (226). The mitochondria from BAT also appear very different from isolated skeletal muscle mitochondria where the cristae have a rather twisted tubular appearance (111). However, mitochondria from either warm- or cold-acclimated rats look very different from isolated BAT

mitochondria which have been tightly recoupled by incubation with ATP, carnitine and CoA (28). The recoupled mitochondria display an orthodox conformation with a matrix largely increased in volume, very little intracristal space and the cristae arranged in parallel rows. On the other hand, dinitrophenol or palmitic acid uncoupled BAT mitochondria are characterized by a condensed matrix and enlarged intracristal space (28) which are actually the predominant features of the mitochondria reported in this thesis. This is not particularly surprising since BAT mitochondria are known to bind fatty acids during the isolation procedure (112). A relationship between mitochondrial ultrastructure and energy metabolism has already been proposed for liver mitochondria (105). Altough there is little doubt that various ultrastructural changes occur on varying the energy state of the mitochondria, the appearance of the mitochondria at the level of the electron microscope most probably reflects osmotic changes.

2. Size distribution of BAT mitochondria from warm- and cold-acclimated rats

Method: Mitochondria were isolated from the BAT of 6 W-A and 15 C-A rats that had been acclimated at either 28°C or 4°C for 4 weeks. The weights of the animals at the time of the experiment were: C-A 326 ± 2 g, W-A 359 ± 3 g. The mitochondrial size distribution was estimated as described previously (Methods, section II-F).

Results and discussion: Figure 17 shows the total volume contributed

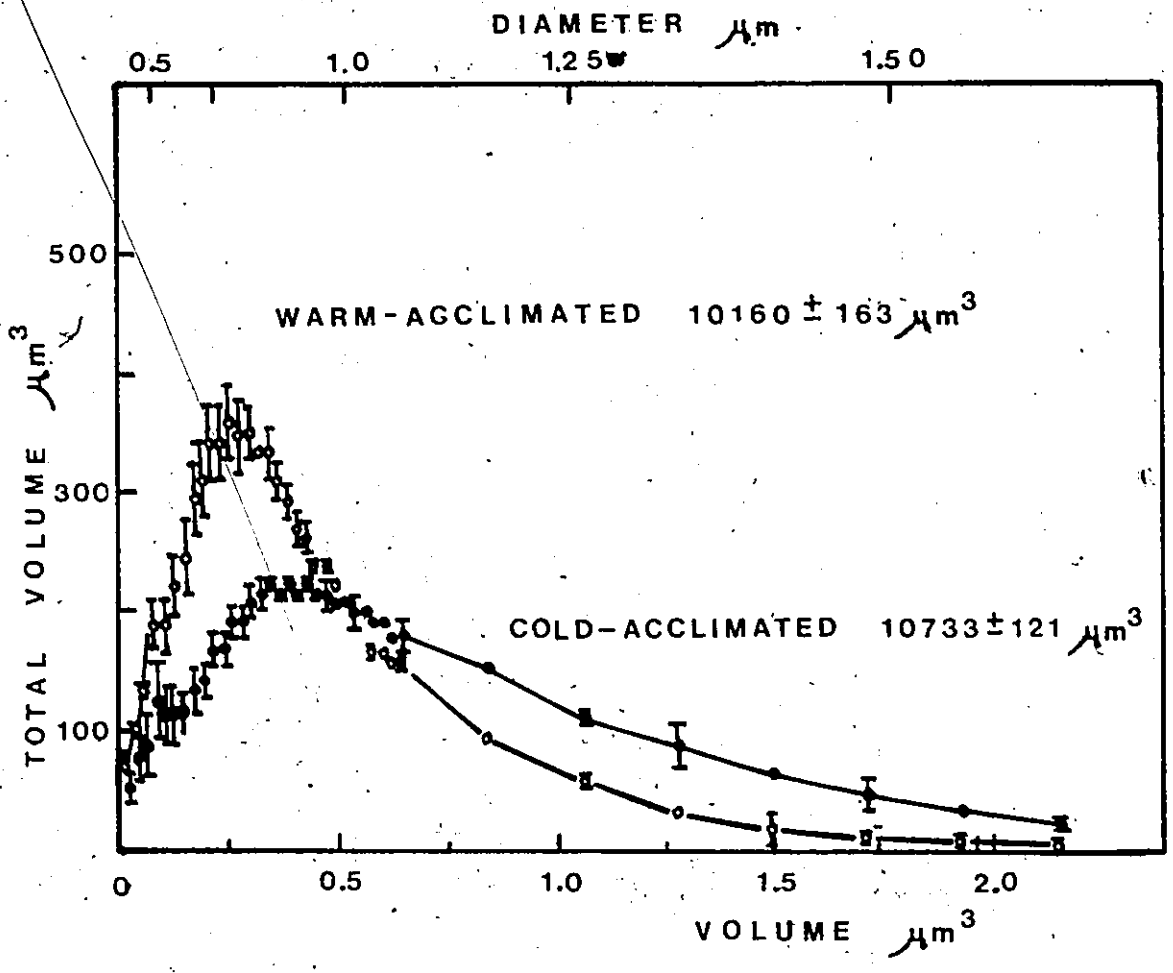

FIG. 17. Size distribution of BAT mitochondria from warm-(WARM) and cold-(COLD) acclimated rats.

This figure shows the total volume contributed by mitochondria of sizes from 0.0218 μm^3 to 2.18 μm^3. The total volume of mitochondria counted in W-A and C-A rats is the same (10 160 ± 163 μm^3 W-A, 10733 ± 121 μm^3 C-A; $n = 3$, p = N.S.). The protein concentrations of mitochondrial suspensions from W-A rats and C-A rats were 0.62 ± .06 μg/ml and 0.70 ± .08 μg/ml respectively.

The volumes shown are the means ± S.E. of 3 separate experiments. When no standard error is shown, the error is too small to be shown separately from the point indicating the mean.

by mitochondria of sizes from 0.0218 μm^3 to 2.182 μm^3. Isolated BAT mitochondria from the C-A rats are larger than those from the W-A rats. A significantly smaller volume ($p < 0.05$ or less) is present in mitochondria of volume 0.11-0.44 μm^3 (diameter 0.6-1.0 μm) in the C-A rats and a significantly larger volume is present in mitochondria of volume 0.59-2.18 μm^3 (diameter 1.2-1.6 μm). It is then clear that C-A rats have fewer mitochondria of small diameter and more mitochondria of larger diameter than do W-A rats. The diameter of the mitochondria corresponds to that observed on the electron micrographs. In the literature, a mean mitochondrial volume of 0.3 μm^3 from the brown fat of newborn guinea pigs has been calculated from electron micrographs (9). This mitochondrial volume is also known to increase shortly after birth or cold acclimation (226). As discussed in more detail in appendix A, the Coulter Counter technique appears very reliable for determining the number and size of mitochondria although in this type of comparative work, one must assume no significant difference in the electrical resistivity of the mitochondria from the cold- and warm-acclimated rats.

Conclusions: Brown adipose tissue mitochondria are altered upon cold acclimation. Mitochondria from cold-acclimated rats are larger and have an altered internal structure. They have an increased surface area of inner membranes resulting from an increased concentration of cristae and a more organized arrangement. Most probably, the increase in the concentration of proton conductance pathways is linked to the increase in mitochondrial membrane material observed here and contributes to the enhanced capacity of the tissue for heat production in C-A rats.

The availability of the sites for purine nucleotide binding is

certainly also a factor to consider. For example, the extensive condensation of the matrix and inner membranes of the mitochondria may have an *in vitro* reducing effect on the extent of binding of the purine nucleotides. Possibly, a combination of both the increase in concentration and availability of binding sites is responsible for the large increase in purine nucleotide binding to BAT mitochondria of C-A rats.

C. DIFFERENCES IN FUNCTIONAL CHARACTERISTICS BETWEEN BAT MITOCHONDRIA OF C-A AND W-A RATS

The results observed in the two previous sections indicated that BAT mitochondria have a higher concentration of purine nucleotide binding sites which are believed to correspond to a higher amount of proton conductance pathways. There was an increase in both purine nucleotide binding and in the proportion of the polypeptide of 32 000 M.W. in BAT mitochondria of C-A rats.

In hamster BAT mitochondria, it was demonstrated that the proton conductance pathway possesses an effective proton current which is proportional to the proton electrochemical gradient and dependent on exogenous purine nucleotides (199, 200). The rate of respiration of the mitochondria is in turn dependent on the magnitude of the proton electrochemical gradient in an inverse-type relationship (195) (see Fig. 7). The measurement of the proton electrochemical gradient appears to provide a valid indication of the mitochondrial "energy potential". The increase in the concentration of proton conductance pathways (as measured by purine nucleotide binding) in mitochondria of C-A rats reported in this thesis should then result in an enhancement of proton cycling across the

inner membrane and hence, in an increased thermogenic capacity.

Objective: The purpose of this work was to compare the proton electrochemical gradient and respiratory capacity of BAT mitochondria from W-A and C-A rats and to correlate them with the extent of binding of purine nucleotides to the mitochondrial membranes.

1. Proton electrochemical gradient of isolated mitochondria from W-A and C-A rats

Method: Proton electrochemical gradient was measured as originally described by Nicholls (197, 200) and modified by Azzone et al (5). The proton electrochemical gradient of the inner membrane is defined by the following relationship: $\Delta p = \Delta E - 59\ \Delta pH$, where ΔE is the membrane potential and ΔpH the transmembrane pH gradient. Basically, the method involves the determination of the pH gradient and the membrane potential via the distribution of radioactively labelled weak bases and acids and of rubidium in presence of valinomycin between the matrix phase and the incubation medium. The theory underlying the technique and an example of calculations are given in appendix C.

Mitochondria from BAT from 3-5 W-A rats and 1-2 C-A rats were isolated as usual and resuspended in the isolation medium at a protein concentration of 10-15 mg/ml. All determinations were carried out in Eppendorf microcentrifuge tubes at room temperature in a final volume of 1.0 ml. The basic incubation medium contained 100 mM sucrose, 5.0 µM rotenone, 10 mM disodium-DL-α- glycerophosphate, 10 µM {^{14}C} - methylammo-

nium chloride (0.15 µCi/ml), 10 mM choline chloride, 10 mM glycyl-glycine, 0.5 mM KCl, 0.5 µM valinomycin, 10 µM {^{3}H} - acetate (1.5µCi/ml), 10 µM {^{86}Rb} -rubidium chloride (0.03 µCi/ml at pH 7.1) The pH was ajusted with either HCl or Tris base.

The mitochondria were added (final protein conc. 0.5-0.8 mg/ml) and 5 minutes were allowed for equilibration. The tubes were then centrifuged in the Eppendorf microcentrifuge 3200 for two minutes. The supernatants were carefully removed and the bottom of the tubes containing the mitochondrial pellets cut and placed in a vial containing 1.0 ml NCS. The vials were then incubated overnight at 50-55^{o}C. Then, 0.05 ml of a 10% ascorbic acid solution was added, followed by 10 ml of toluene-PPO (0.5%). The samples were then counted for ^{3}H, ^{14}C and ^{86}Rb. The matrix volume was determined according to Malamed and Recknagel (181) by incubating 2-4 mg mitochondrial protein in centrifuge tubes in the basic incubation medium supplemented with ^{14}C-sucrose (0.3 µCi/ml). The ratio mitochondrial suspension/incubation medium was kept the same as in the proton electrochemical gradient measurements. After 5 minutes, the tubes were centrifuged for 1 hour at 16 300 xg. The supernatant solutions were poured off and the inner walls of the tubes blotted with paper. The pellets were then used for wet weight - dry weight determinations. The pellets were dried to constant weight in the oven at 80^{o}C, usually overnight in the presence of desiccant. To the dried pellets, 1.0 ml NCS was added; the tubes were capped and incubated at 55^{o}C until complete dissolution of the pellets. An aliquot was then taken, put into a vial to which were added 0.05 ml of a 10% ascorbic acid solution and 10 ml of toluene-PPO (0.5%) and counted (^{14}C).

In a first section of the work, the influence of the incubation

conditions (as defined in the legend to Fig. 18) on the proton electrochemical gradient of mitochondria from W-A and C-A rats was studied. For this purpose, a total of 3 C-A rats and 9 W-A rats weighing respectively 476 ± 19 g and 544 ± 19 g were used. They had been acclimated at either 28°C or 4°C for 12-13 weeks.

In a second section of this work, the proton electrochemical gradient of mitochondria incubated in presence of 0.5% albumin (fatty acid free) was estimated as a function of an increasing concentration of exogenous GDP (0-1 000 μM). For this purpose, 12 C-A rats and 30 W-A rats weighing respectively 345 ± 9 g and 395 ± 17 g were used. They were acclimated at either 28°C or 4°C for a period of 4-6 weeks.

Results and discussion: Freshly isolated mitochondria from either warm- or cold-acclimated rats are unable to maintain a significant proton electrochemical gradient ($\Delta p = 0$) during substrate oxidation (Fig. 18). Both populations of mitochondria are completely de-energized. The inclusion of albumin or GDP in the incubation medium allows the build-up of a proton electrochemical gradient of 20-80 mV. The proton electrochemical gradient is slightly higher in the mitochondria from the W-A rats. Upon addition of GDP, the Δp values are 76 ± 2 mV for the W-A and 61 ± 8 mV for the C-A (p = N.S.) Upon addition of albumin, the Δp values are 39 ± 0.3 mV for the W-A rats and 21 ± 2 mV for the C-A rats ($p < 0.02$). Maximum Δp (200 mV) is however maintained when both GDP and albumin are present together in the incubation medium. The maximum Δp obtained is the same in the two types of animals. However, when the proton electrochemical gradient is determined as a function of exogenous GDP concentration, it can be seen that a higher concentration of GDP is required by BAT

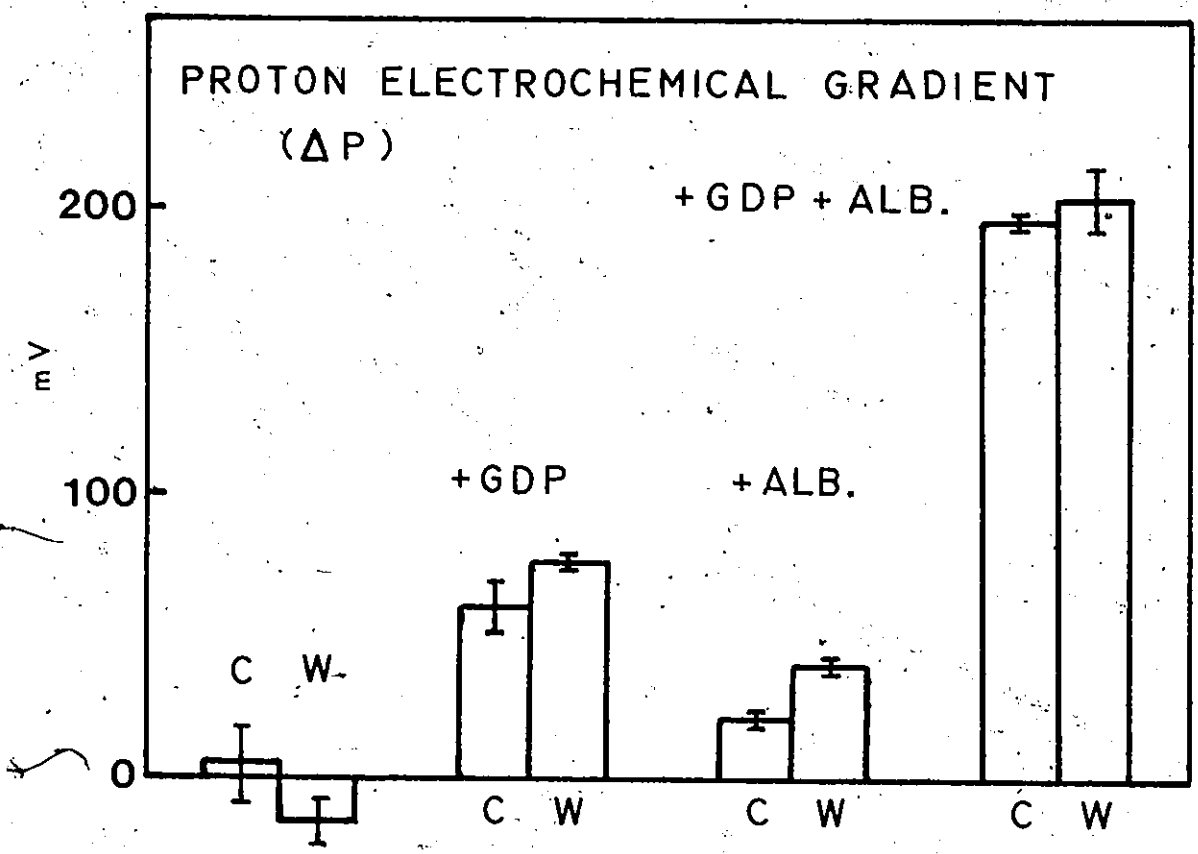

FIG. 18. Proton electrochemical gradient of BAT mitochondria from W-A and C-A rats.

Mitochondria (0.5 - 0.8 mg/ml were incubated at room temperature in the basic medium (see Method) with the following additions: (a) no addition, (b) 1 mM GDP, (c) bovine serum albumin (fatty acid free), (d) 1 mM GDP and 0.5% albumin.

The values are the means ± S.E. of 3 separate experiments. The matrix volumes were estimated to be: 0.50 ± 0.02 µl/mg mitochondrial protein W-A; 0.64 ± 0.09 µl/mg C-A; p = N.S.

mitochondria from C-A rats to reach maximum Δp (Fig. 19). The Δp obtained in the latter experiment did not reach the same value observed previously and is also lower than corresponding literature data. This is due to an overestimation of the matrix volume in this experiment. It can be easily verified by comparing the matrix volume data given under Fig. 18 and 19. Although the absolute numbers are reduced, the relation Δp vs GDP concentration is not affected since all the results are actually divided by the same volume determination. The matrix volumes obtained are nevertheless within the range observed by others (204, 278). The concentration needed to induce half maximal energization is about 4 μM for the W-A rats and 12 μM for the C-A rats. The concentration needed to induce half maximum energization in mitochondria from warm-acclimated rats (4 μM) is equivalent to the binding affinity constant observed in section I-A, (6 μM). Fig. 19 is actually equivalent to an enzyme-catalyzed reaction where the dissociation of the enzyme-substrate complex is extremely slow or does not occur and the K_m becomes the dissociation constant. However, this type of curve (Fig. 19) does not differentiate between binding affinity and number of binding sites for purine nucleotides. However, since it is known that the binding affinity of purine nucleotides for mitochondria of C-A rats is not different from that of W-A rats, (see section I-A) it can be concluded that the increase in the concentration needed to induce half maximum energization in mitochondria of C-A rats is due to an increase in the number of binding sites.

The same requirement for a higher exogenous GDP concentration is also observed for the mitochondria of the C-A rats to achieve a maximum ΔpH (Fig. 20) but not for them to reach a maximum membrane potential (Fig. 21). The major influence of GDP is then to inhibit the proton

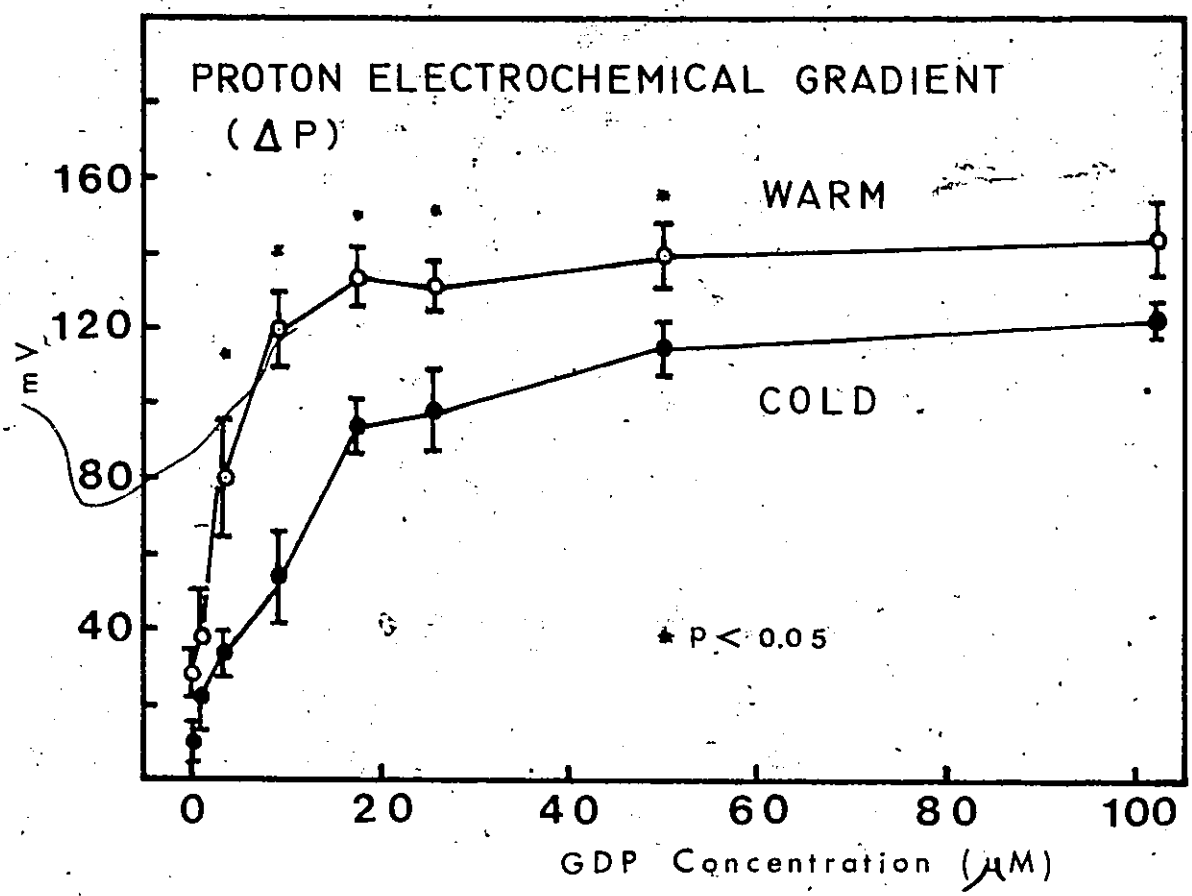

FIG. 19. Proton electrochemical gradient of BAT mitochondria of W-A and C-A rats as a function of exogenous GDP concentration.

Mitochondria (0.5 - 0.8 mg/ml) were incubated at room temperature in the basic medium (see Method) supplemented with 0.5% bovine serum albumin (fatty acid free) and with 0 - 100 μM GDP.

The values are the means ± S.E. of 6 separate experiments. The mitochondrial matrix volumes were estimated to be 1.15 ± 0.08 μl/mg W-A; 1.83 ± 0.13 μl/mg C-A; $p < 0.005$.

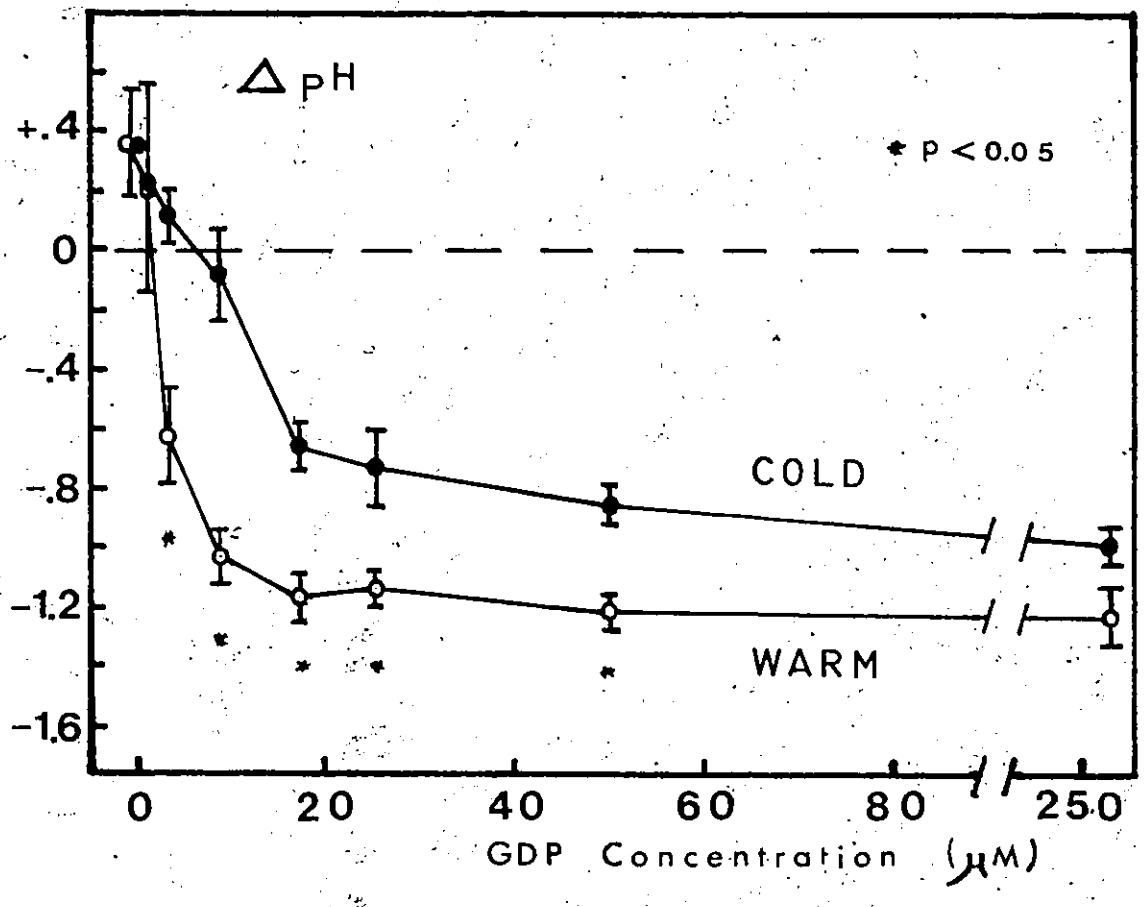

FIG. 20. Transmembrane ΔpH of BAT mitochondria of W-A and C-A rats as a function of exogenous GDP concentration.

The incubation conditions and experimental details are the same as under Fig. 19.

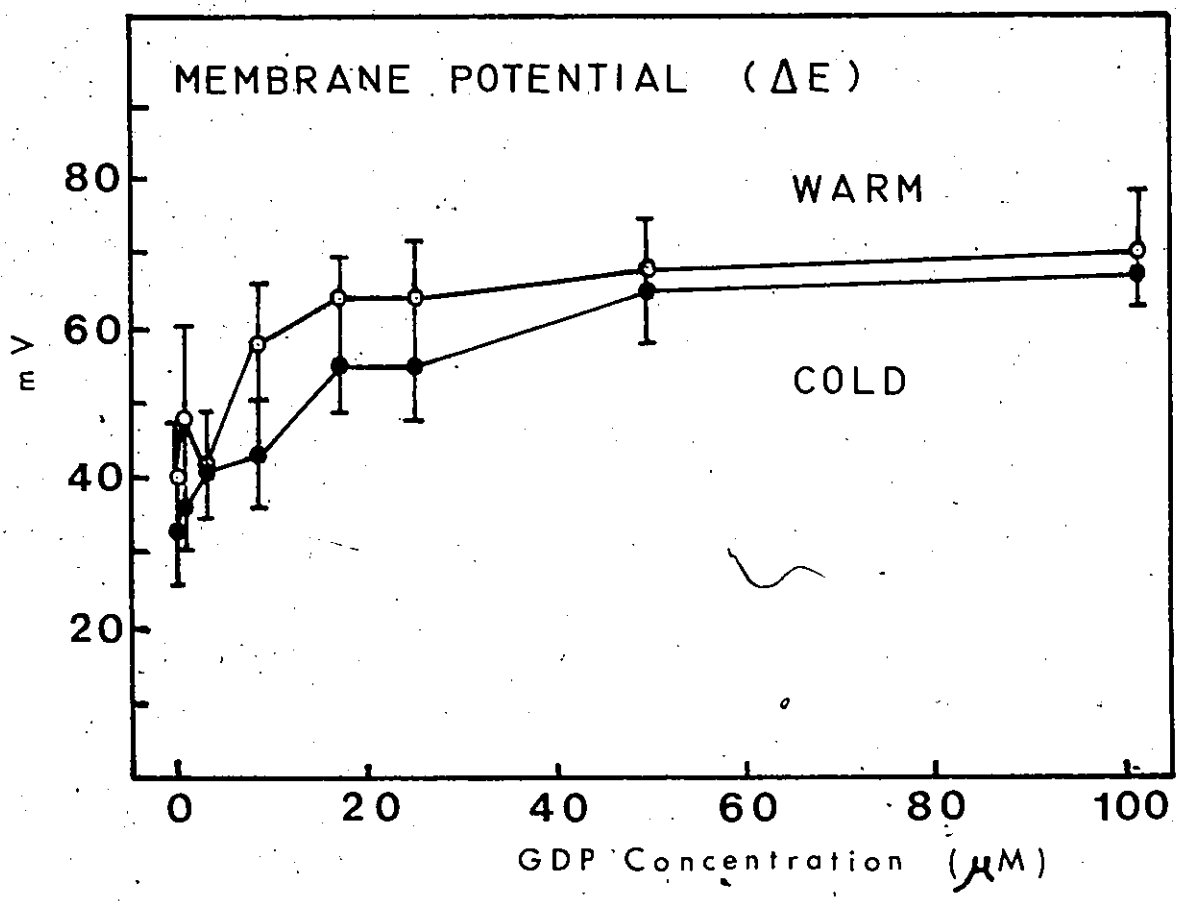

FIG. 21. Membrane potential of BAT mitochondria of W-A and C-A rats as a function of exogenous GDP concentration.

The incubation conditions and the experimental details are the same as under Fig. 19.

leakage into the matrix allowing the built-up of the proton gradient. The action of albumin on the other hand, may be ascribed to the removal of bound fatty acids.

Mitochondria from cold-acclimated animals are known to be similar to those of a hibernator. As such, freshly isolated mitochondria from cold-adapted hamsters are also completely de-energized ($\Delta p=0$) at pH 7.1 and can only reach a maximum Δp (220 mV) in presence of both albumin and GDP (200). Intermediate values from 60-130 mV are obtained when the incubation medium is supplemented with either GDP or albumin alone (200)

Pedersen and Flatmark compared 6 day cold-stressed guinea pigs and their warm-readapted controls using the redox level of the cytochrome b complex as an internal probe for the estimation of the energy state of the mitochondrial membranes. They showed the complete de-energization of the mitochondria from both preparations at pH 7.1 (212). A higher ATP requirement for the energization of the mitochondria from the cold-stressed guinea pigs was also reported although the concentration needed was a 100 folds higher than those reported in this thesis. The recoupling effect of GDP or ATP is known to be similar (196). Part of the difference may be explained by the absence of albumin and atractyloside in their incubation medium.

In conclusion, the increase in purine nucleotide binding of the mitochondria from the C-A rats is associated with a corresponding change in the control of the proton electrochemical gradient of the mitochondria.

2. Respiratory rates of BAT mitochondria of warm- and cold-acclimated rats

Method: A Clark oxygen electrode (Yellow Spring Instrument's Biological Oxygen monitor) was employed for the measurement of O_2 consumption. The incubation temperature was maintained at 23-25°C throughout. Two different incubation media were used for the experiment. Medium A was exactly the same as used for the measurement of the proton electrochemical gradient and contained: 100 mM sucrose, 10 mM choline chloride, 10 mM glycylglycine, 0.5 mM KCl, 0.5 µM valinomycin, 5.0 µM rotenone, 10 µM methylammonium chloride, 10 µM acetate, 10 µM RbCl, 0.5% bovine serum albumin (fatty acid free) pH 7.1. 10 mM disodium-DL-α-glycerophosphate was used as substrate.

Medium B, on the other hand, contained: 75 mM NaCl, 10 mM TES, 1 mM EGTA, 1 µM rotenone and 2.0 mg/ml bovine serum albumin (fatty acid free) pH 7.0. 10 mM succinate or disodium-DL-α-glycerophosphate was used as substrate. The medium was equilibrated with air before use and the O_2 content was assumed to be 490 nmoles O_2/ml (200).

Mitochondria (0.5-1.0 mg) were added to 3.0 ml of incubation medium, followed by GDP at various concentrations (as specified under Table I and Fig. 23) and mCCP (7 µM). A typical experiment is depicted in Fig 22.

The respiratory rates are expressed in nmoles O_2 consumed / min.mg mitochondrial protein and are given in Table I. In Fig. 23, the results are expressed as the ratio of coupled (respiration in presence of GDP) to uncoupled (in presence of mCCP) respiration of BAT mitochondria as a function of exogenous GDP concentration. For these experiments, BAT mitochondria were isolated from 12 W-A and 6 C-A rats weighing respectively 460 ± 10 g and 386 ± 15 g. They had been acclimated at either 28°C or 4°C

for 4-8 weeks. The mitochondria were resuspended in the isolation medium, kept on ice and used as soon as possible after the isolation.

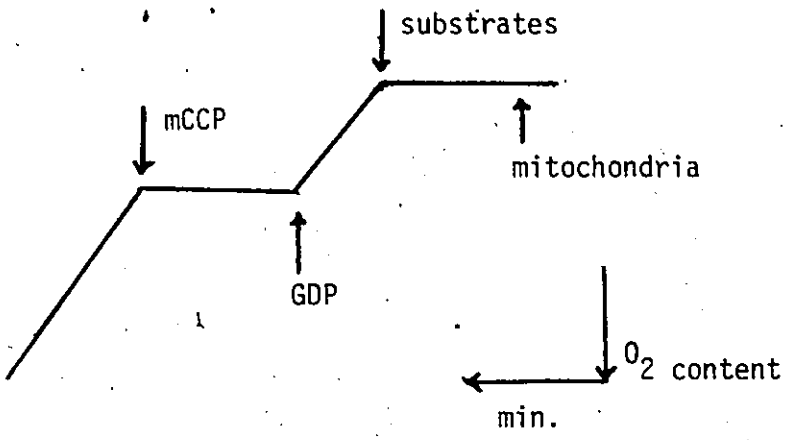

Fig. 22. Measurement of the respiratory rates of isolated mitochondria from brown adipose tissue.

Results and discussion: The rates of controlled respiration are dependent on the proton electrochemical gradient (195). The chemiosmotic theory actually considers the respiratory control as a feedback inhibition induced by the proton electrochemical gradient. As such, the change in the purine nucleotide control of the Δp observed in the previous section should also be reflected by a corresponding change in the rate of respiration. It is indeed the case. In Fig. 23, the ratio of the rate of respiration in the absence of mCCP to the rate of respiration in its presence (uncoupled respiration) is plotted against the concentration of added GDP. The lower the ratio, the greater the state of coupling of the mitochondria. A ratio of 1.0 is indicative of totally uncoupled

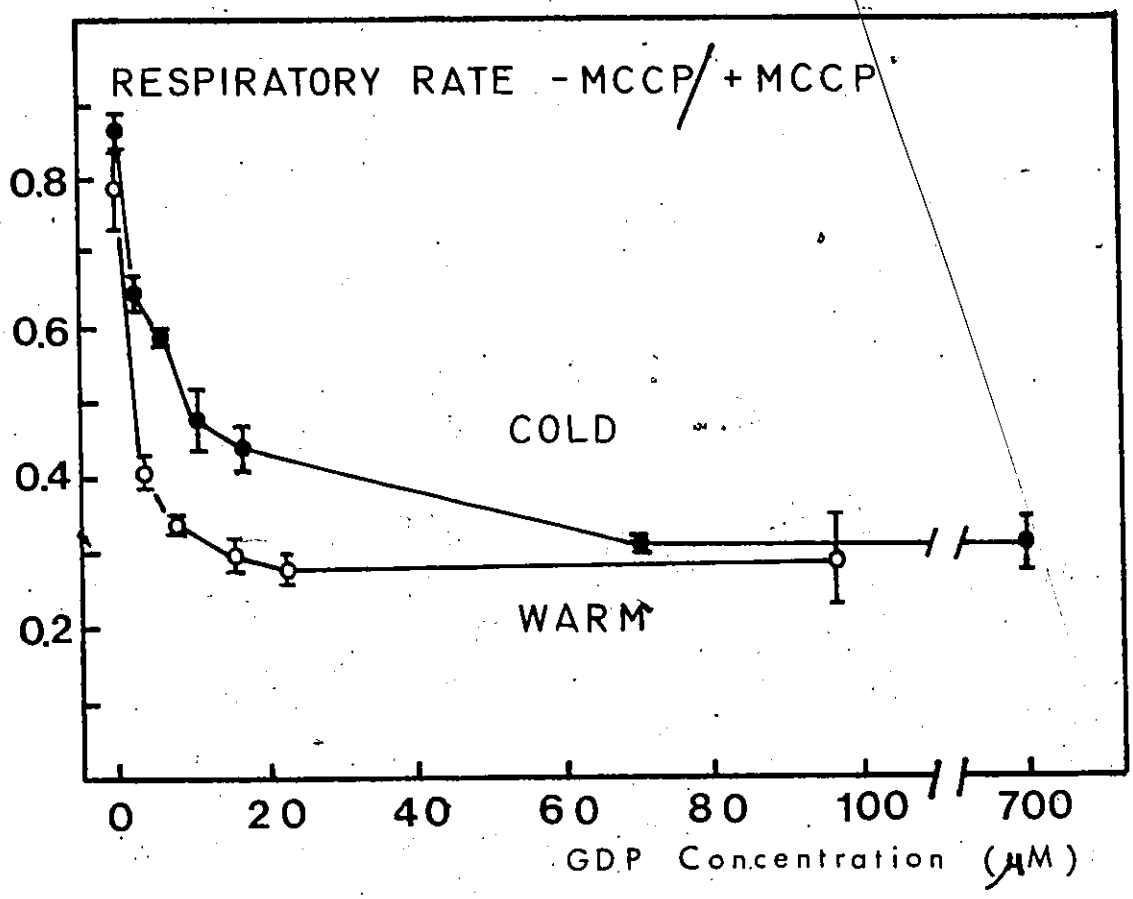

FIG. 23. Ratio of coupled to uncoupled respiration of BAT mitochondria of W-A and C-A rats as a function of exogenous GDP concentration.

Mitochondria (1 mg) are incubated in 3.0 ml of medium A (see Method) at room temperature and the respiratory rates measured after addition of various concentration of GDP and mCCP (7 μM). The respiratory ratios were calculated from the respiratory rates thus obtained. The results are the means ± S.E. of 3 separate experiments.

mitochondria. The mitochondria from the C-A rats have the same requirement as observed previously for a higher GDP concentration to achieve a maximally coupled state (Fig. 23).

Table I gives the respiratory rates and respiratory ratios for warm- and cold-acclimated rats using succinate or α-glycerophosphate as substrate. The mitochondria from the C-A rats have a higher respiratory rate when oxidizing succinate but not when respiring with α-glycerophosphate. The respiratory rates of both warm- and cold-acclimated rats are significantly reduced in presence of 1 mM GDP but no difference can be observed between them. The rates of respiration of the C-A rats do not correlate with the increased amount of proton conductance pathways. The 1.7 fold increase in respiratory rate (succinate) does not match the 3-4 fold increase in the concentration of the proton conductance sites and no change in respiratory rates is observed with α-glycerophosphate. The increase in the concentration of the proton conductance pathways in the C-A rats does not result in an increased thermogenic capacity of the mitochondria. An increase in specific activity of succinate dehydrogenase in isolated mitochondria of C-A rats has been reported (254) and is most probably responsible for the increase in the respiratory rate observed in this work. On the other hand, the specific activity of the α-glycerophosphate dehydrogenase is unchanged in mitochondria from C-A rats (254), in agreement with the unchanged rate of respiration observed here.

The respiratory ratios of mitochondria from W-A and C-A rats are also informative. The ratios of the respiratory rates αGP/mCCP or succinate/mCCP show that mitochondria from W-A rats are slightly more coupled than those of C-A rats in absence of purine nucleotides. A complete lack of stimulation of respiration by the proton translocator

TABLE I. Respiratory rates of BAT mitochondria from W-A and C-A rats.

Mitochondria (0.5 - 1.0 mg) were incubated in 3.0 ml of medium B (see Method) at 23-25°C with either succinate or α-glycerophosphate as substrate. The respiratory rates were recorded after addition of 10 mM substrate, 1 mM GDP and 7 μM mCCP in that order as described in Fig. 22. The results are expressed as nmoles O_2 consumed / min.mg mitochondrial protein and are the means ± S.E. of 3 separate experiments.

	n	+ α-GP	+ GDP	+ mCCP	n	+ succ.	+ GDP	+ mCCP
C-A	3	200 ± 11	69 ± 1	197 ± 10	3	295 ± 24	88 ± 5	302 ± 17
W-A	3	244 ± 19	87 ± 7	282 ± 16	3	175 ± 5	97 ± 9	197 ± 3
p <		NS	NS	.02		.01	NS	.005

Ratio respiratory rates: substrate (α-GP or succ.) / mCCP GDP / mCCP

C-A	6	0.997 ± 0.020	0.322 ± 0.02
W-A	6	0.875 ± 0.015	0.400 ± 0.05
p <		0.001	NS

mCCP gives a ratio of 1.0 and is indicative of totally uncoupled mitochondria. Mitochondria of C-A rats are totally uncoupled. However, the whole population of mitochondria from W-A rats is not tightly coupled. The ratio of coupled respiration (GDP) to uncoupled (mCCP) respiration is 0.4 ± .05. This is much lower than the ratio obtained in absence of GDP which reaches 0.8-0.9. These results agree with the proton electrochemical gradient observed in Fig. 18 where the Δp becomes maximum only after incubation with albumin and GDP and is not significantly different between the two types of animals. In absence of GDP, a low Δp is sustained, slightly higher in the W-A rats but nevertheless insufficient to induce full respiratory control. The difference in the ratio of respiration αGP/mCCP or succinate/mCCP between C-A and W-A rats may possibly be ascribed to the presence of a low amount of purine nucleotides in the incubation medium resulting from the breakage of some mitochondria or the "leakage" of nucleotides from them. Mitochondria from W-A rats have a very low concentration of purine nucleotide binding sites and the presence of a low amount of exogenous nucleotides may induce the recoupling of a small fraction of the mitochondrial population resulting in the lowering of respiratory ratios. Most likely, isolated mitochondria from either warm- or cold-acclimated rats, in complete absence of purine nucleotides (i.e with their proton channels open) are uncoupled. By making mitochondrial preparations very rapidly, Cannon has been able to isolate hamster and lamb BAT mitochondria that still possess some uncoupler-sensitive respiration. This indicates that, at least in certain cases, the de-energized state of freshly isolated mitochondria may be a consequence of the isolation technique (32).

Similar respiratory ratios have been obtained by other laboratories.

Ratios of respiration in presence and absence of FCCP of 0.62-0.79 have been reported for warm-adapted rats and guinea pigs and 1.0 for cold-adapted hamsters, guinea pigs and rats (114, 203) . The respiratory ratio -FCCP/ FCCP in absence of purine nucleotide in liver mitochondria is 0.11 when using succinate as substrate, 0.2 with α-ketoglutarate and palmitoylcarnitine and 0.5 with α-glycerophosphate (81).

Direct comparison of the respiratory rates reported in this thesis with those of the literature is not possible because of the large variety of experimental conditions and animal species used. However, an increased respiration with succinate has been observed in 10 day cold-stressed (4) and newborn guinea pigs (45) but the endogenous respiration of mitochondria from warm- and cold-stressed guinea pigs was not different (4). An increase in state 4 respiration of BAT mitochondria of C-A rats oxidizing α-glycerophosphate has been observed (203). The reasons for the discrepancy between these results and those presented in this thesis are unknown. However, the addition of FCCP did not abolish the difference between the respiratory rates of mitochondria from W-A and C-A rats (203) indicating that proton re-entry into the matrix is not the major factor responsible for this increase.

Based on criteria such as P/O, ADP/O ratio or respiratory stimulation by ADP or Ca^{++}, a loosening of coupling of isolated BAT mitochondria has been observed during the thermogenically active state (newborn and C-A animals) (43, 81). The interpretation of the results obtained when ADP is used to test for respiratory control is difficult since ADP serves as a recoupling nucleotide and as a phosphate acceptor at the same time. This difficulty is overcome when Ca^{++} is used to test for respiratory control. Stimulation of respiration by ADP or Ca^{++} has not been checked

in this thesis but would not be expected to occur in warm- and cold-acclimated rats because the proton electrochemical gradient is not high enough under the experimental conditions used to induce respiratory control. A Δp of about 165-180 mV is needed in order to get respiratory control (201). A pH of 7.1 for the incubation medium was used in this work while previous workers used a pH of 6.8. A low pH decreases significantly the amount of nucleotides required for recoupling the mitochondria (200). There is also a difference in the pH dependence of the energization process between cold-stressed and warm-readapted guinea pigs (212). A higher energy potential is created in mitochondria from warm-readapted guinea pigs in lowest pH range (6.0 - 7.0).

The concentration of adenosine phosphates in the extramitochondrial space in newborn guinea pig BAT is reported to be 18 nmoles/mg mitochondrial protein (229). This value was obtained by total extraction of adenosine phosphates from the tissue. The actual concentration of free nucleotides available for recoupling is probably lower. The concentrations of nucleotides necessary for recoupling the mitochondria of W-A and C-A rats are estimated from figures 18 and 23. They are 12 nmoles/mg mitochondrial protein and 30-36 nmoles/mg mitochondrial protein for W-A and C-A rats respectively. When compared with a cytoplasmic concentration of adenosine phosphates of 18 nmoles/mg mitochondrial protein (229), it is clear that mitochondria of W-A rats are probably fully coupled in vivo while those from C-A rats are not.

Conclusions: The increase in purine nucleotide binding of mitochondria from C-A rats is associated with a corresponding change in the control of the proton electrochemical gradient. A higher GDP concentration

is needed in C-A rats to achieve a Δp equivalent to that of W-A rats. Similarly, a higher concentration of GDP is also needed to achieve a maximally coupled state of mitochondria from C-A rats. However, the increase in the concentration of proton conductance sites does not result in an increased total thermogenic capacity (maximum rate of respiration) of the mitochondria from C-A rats. The rates of mitochondrial respiration are more dependent on the rate of substrate oxidation than on the capacity of the mitochondria for proton cycling. The respiratory rates of the mitochondria from either warm- or cold-acclimated rats, coupled or uncoupled are not different when they are oxidizing α-glycerophosphate. The increase in respiratory rates of mitochondria from C-A rats when succinate is used as substrate, may be ascribed to the increase in the specific activity of succinate dehydrogenase (254). A regulatory function must be postulated for the increase in the concentration of proton conductance pathways in the mitochondria of C-A rats.

It is then suggested that the increase in concentration of proton conductance pathways is needed to ensure the uncoupling of mitochondria during the thermogenic active states by raising the number of sites higher than can be blocked by the nucleotides present in the cytoplasm. Thermogenesis would then be dependent upon substrate availability (lipolysis) under β-adrenergic control.

PART II. TIME-COURSE OF THE APPEARANCE DURING ACCLIMATION AND THE DISAPPEARANCE DURING DEACCLIMATION OF THE DIFFERENCES IN BAT MITOCHONDRIA OF C-A RATS

A. ACCLIMATION TO COLD

1. Time-course of the appearance of changes in rat BAT mitochondrial composition during acclimation to cold

In part I, it was shown that cold-acclimated rats have a higher concentration of proton conductance pathways characterized by an increase in both purine nucleotide binding and in the proportion of 32 000 polypeptide. There is also a decrease in two other polypeptides (50 000 and 96 000 M.W.) of unknown function in BAT mitochondria of C-A rats. It is important to establish whether the changes observed in mitochondrial composition parallel the development of the cold-acclimated state if they are to be implicated in a mechanism of nonshivering thermogenesis.

Objective: The purpose of this work is to find out whether the changes in mitochondrial composition observed are related to cold adaptation or secondary to acute changes occurring in response to cold stress.

Method: A large group of young rats recently acquired from the breeder and weighing 150-200 g was divided into 2 groups and placed in individual cages either in the cold (4°C) or warm (28°C) room. Measurements of wet weight and protein content of BAT, purine nucleotide binding and membrane polypeptide composition of isolated mitochondria were made as

described previously on three cold-exposed and three control rats after each of the following times spent in either the cold or the warm room: 1, 6, 12 hours, 1 and 3 days, 1, 2, 4, 6, 8 weeks.

For some of the times (1, 6, 12 hours, 1-3 days), the same mitochondrial preparations were used for the estimation of purine nucleotide binding and polypeptide composition. For other times (1, 2, 4, 6, 8 weeks), different preparations from different groups of rats were used.

Results and discussion: BAT grows during acclimation of rats to cold. The weight of the tissue per unit of body weight increases maximally during the first 7 days and at a slower rate thereafter (Fig. 24). The protein content of the tissue increases to the same extent as the wet weight and follows a similar time course (Fig. 25). These results agree with already published data (240, 279). Mitochondrial proliferation as indicated by an increase in the tissue cytochrome oxidase content is maximum within 14 days of acclimation to cold (130). By 8 days, Smith and Roberts (240) reported a maximum tissue O_2 consumption which did not vary significantly with prolonged cold exposure.

A marked increase in the binding of both GDP (Fig. 26) and ADP (Fig. 27) occurs within one hour of exposure to cold. The amount of binding reaches a maximum after 3-7 days, and thereafter declines to a level about 4 times that of the control animals. The time courses of the changes in polypeptide composition occurring upon acclimation to cold are however different. The increase in the 32 000 M.W. polypeptide is slower. It is apparent at 12 hours, reaches a maximum at about 2 weeks and declines thereafter (Fig. 28). Since the proportion of the peptide in the control warm-acclimated rats also declines in a parallel fashion, the actual

increase in C-A remains fairly constant. The decrease in the 96 000 polypeptide also starts within 12 hours and reaches a maximum at about 2 weeks (Fig. 29). The decrease in the 50 000 polypeptide is apparent only after a week and reaches a maximum at about 2 weeks (Fig. 30).

An increase in purine nucleotide binding occurs within one hour of exposure of rats to cold before any change in polypeptide composition can be detected (compare Fig. 26 and 28). This lack of change in the peptide pattern when there are changes in purine nucleotide binding might have several explanations. One possibility is an initial unmasking, exposing sites that are already present but unavailable for binding. Transformation of purine nucleotide binding precursor proteins is another possibility. It is also possible that the electrophoretic method used to measure changes in the amount of polypeptide may not be sufficiently sensitive to detect small changes.

On prolonged exposure to cold, there is an increase in the proportion of the 32 000 polypeptide accompanied by a further increase in purine nucleotide binding. These results have been very recently confirmed (33, 235). It is also known that the incorporation of amino acids into BAT mitochondria is transiently increased both in vivo and in vitro during acclimation to cold (27).

It may not be only a coincidence that the major polypeptide which increases in amount is one-third the size of the major polypeptide which disappears and that the increase in the former is three times the decrease in the latter (compare Fig. 28 and 29). The mechanism by which a change may be brought about is unknown. It is also not known whether there is any relation between the 96 000 polypeptide reported here and the 103 000 polypeptide isolated by GDP-agarose affinity chromatography (235).

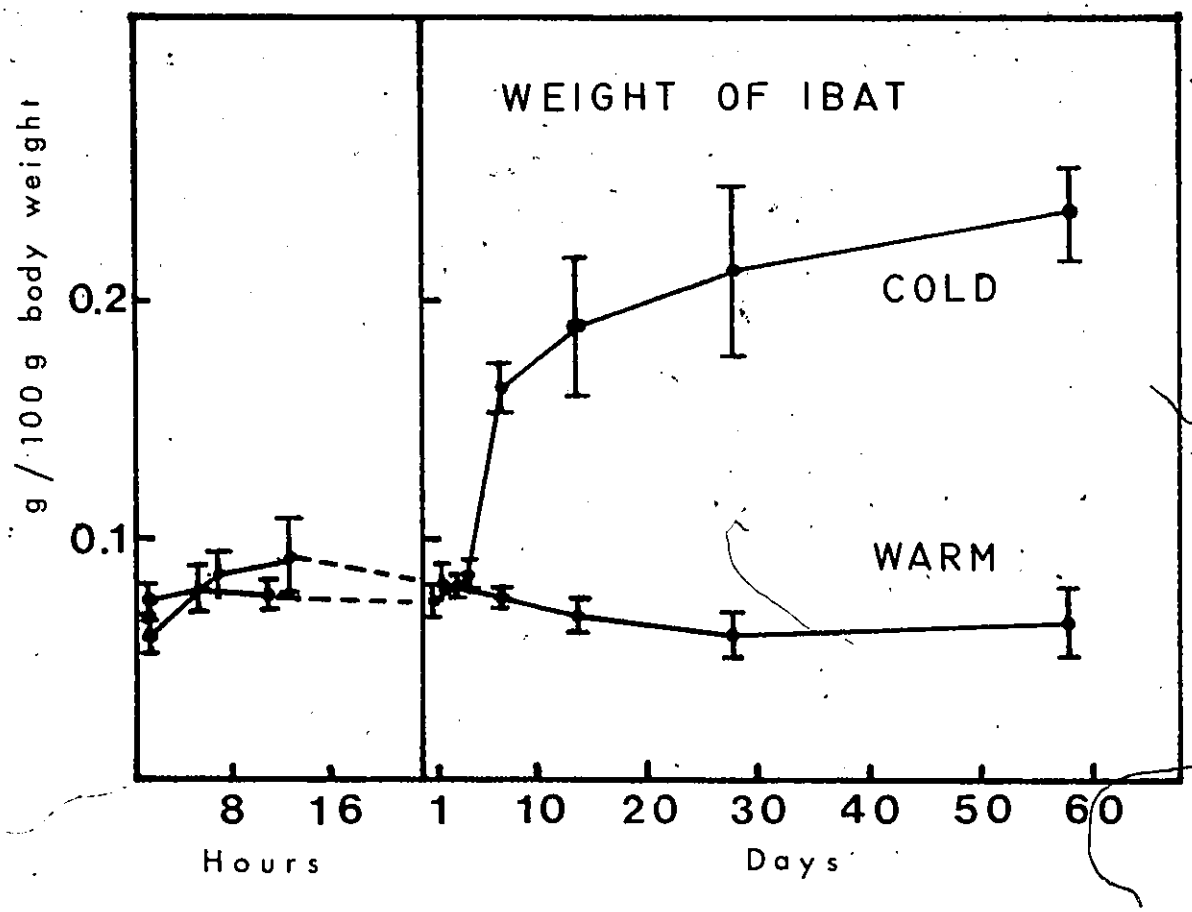

FIG. 24. Growth of interscapular brown adipose tissue (IBAT) during acclimation of rats to cold.

Values are the means ± S.E. of 3-6 observations. Rats were maintained at either 4°C (COLD) or 28°C (WARM) for the times indicated.

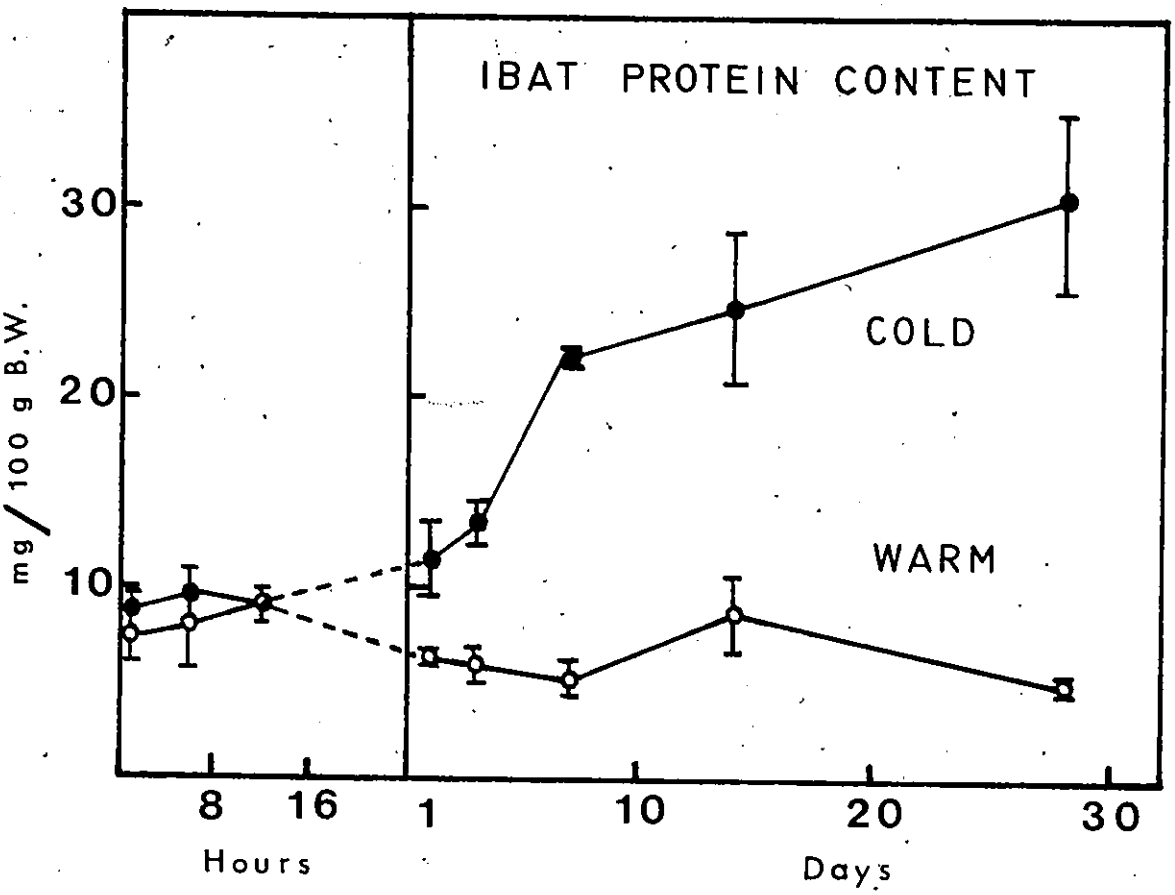

FIG. 25. Protein content of interscapular brown adipose tissue (IBAT) during acclimation of rats to cold.

Rats were maintained at either 4°C (COLD) or 28°C (WARM) for the times indicated. Values are the means ± S.E of 3 observations.

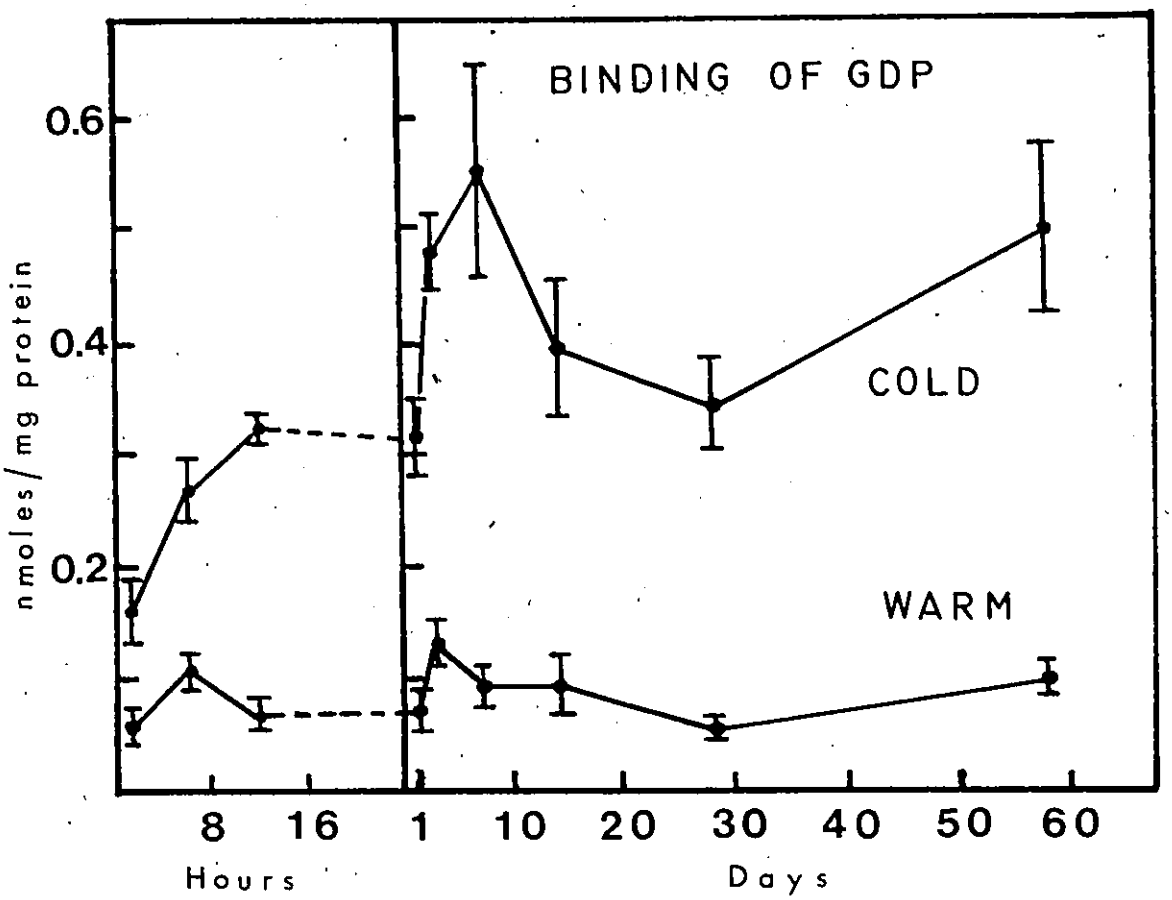

FIG. 26. Binding of GDP by brown adipose tissue mitochondria during acclimation to cold (COLD) in comparison with the binding in control (warm-acclimated (WARM)) rats.

The left hand part of the figure shows changes during the first day and the right hand part shows changes occurring during the subsequent 8 weeks. Values are the means ± S.E. of 3 experiments.

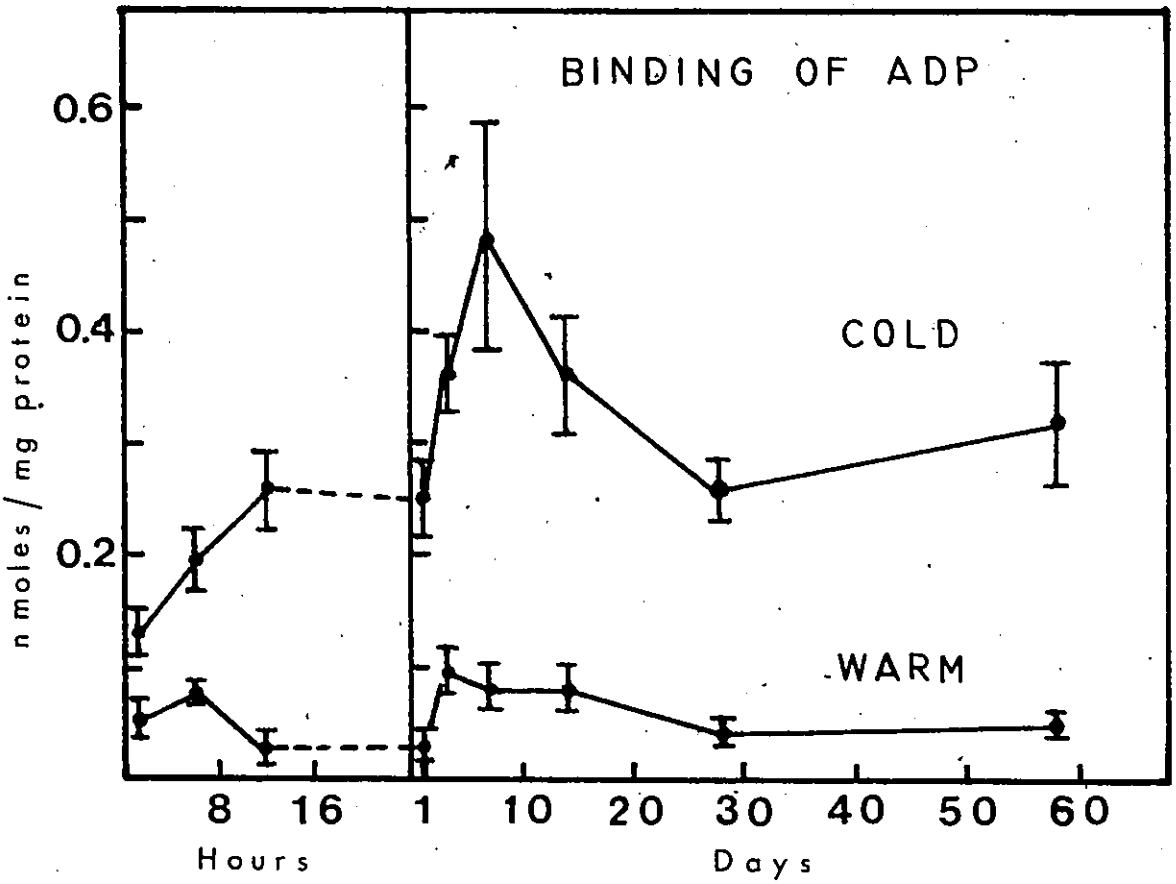

FIG. 27. Binding of ADP by BAT mitochondria during acclimation to cold (COLD) in comparison with the binding in control (warm-acclimated (WARM)) rats.

The time scale is expanded in the left hand portion of the figure to illustrate events occurring during the first 24 hours. Values are the means ± S.E. of 3 experiments.

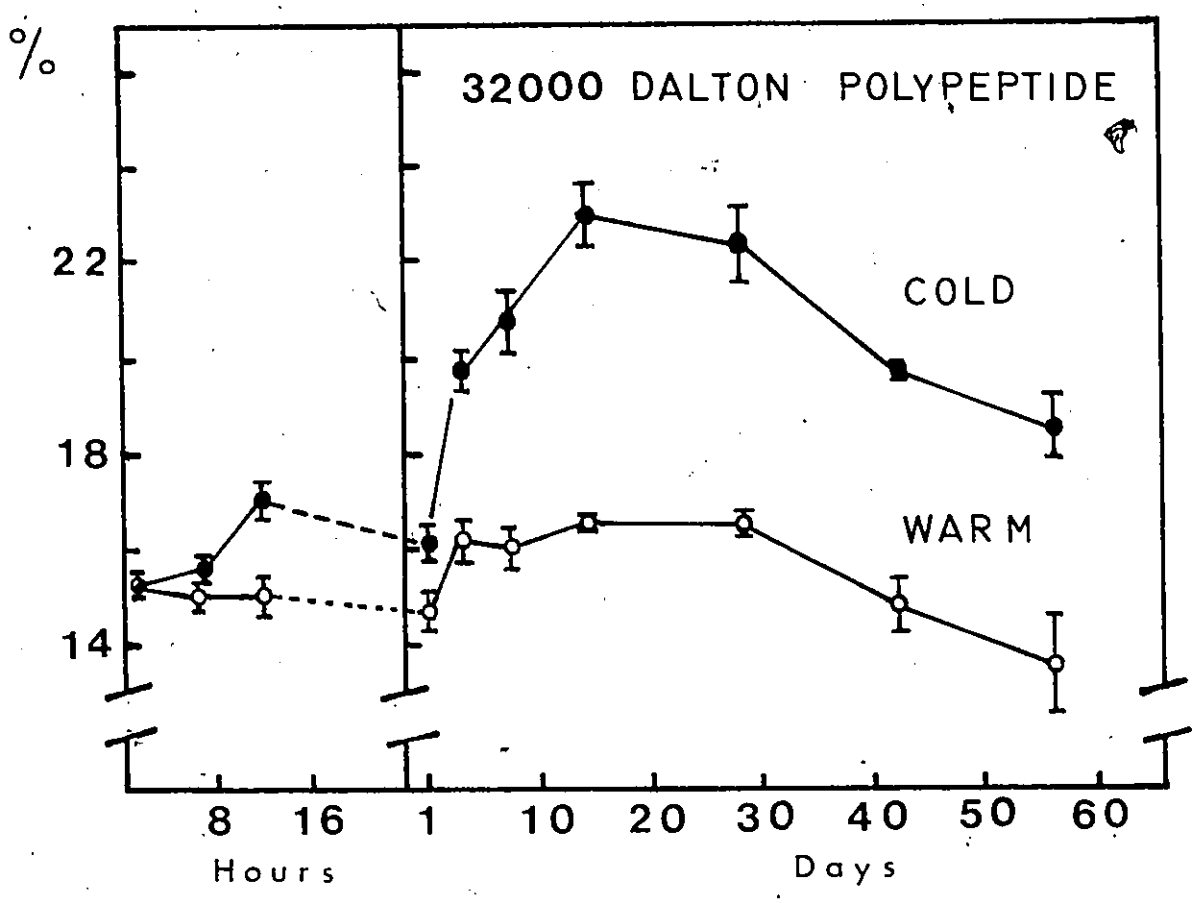

FIG. 28. Proportion of the 32 000 M.W. polypeptide of brown adipose tissue mitochondrial membrane proteins during acclimation of rats to cold.

Rats were kept at either 4°C (COLD) or 28 C°(WARM) for the times indicated. For each point, the number of rats studied is two or three. The left hand part of the figure shows changes during the first 24 hours and the right-hand part shows changes occurring during the subsequent 8 weeks. Values are means ± S.E.

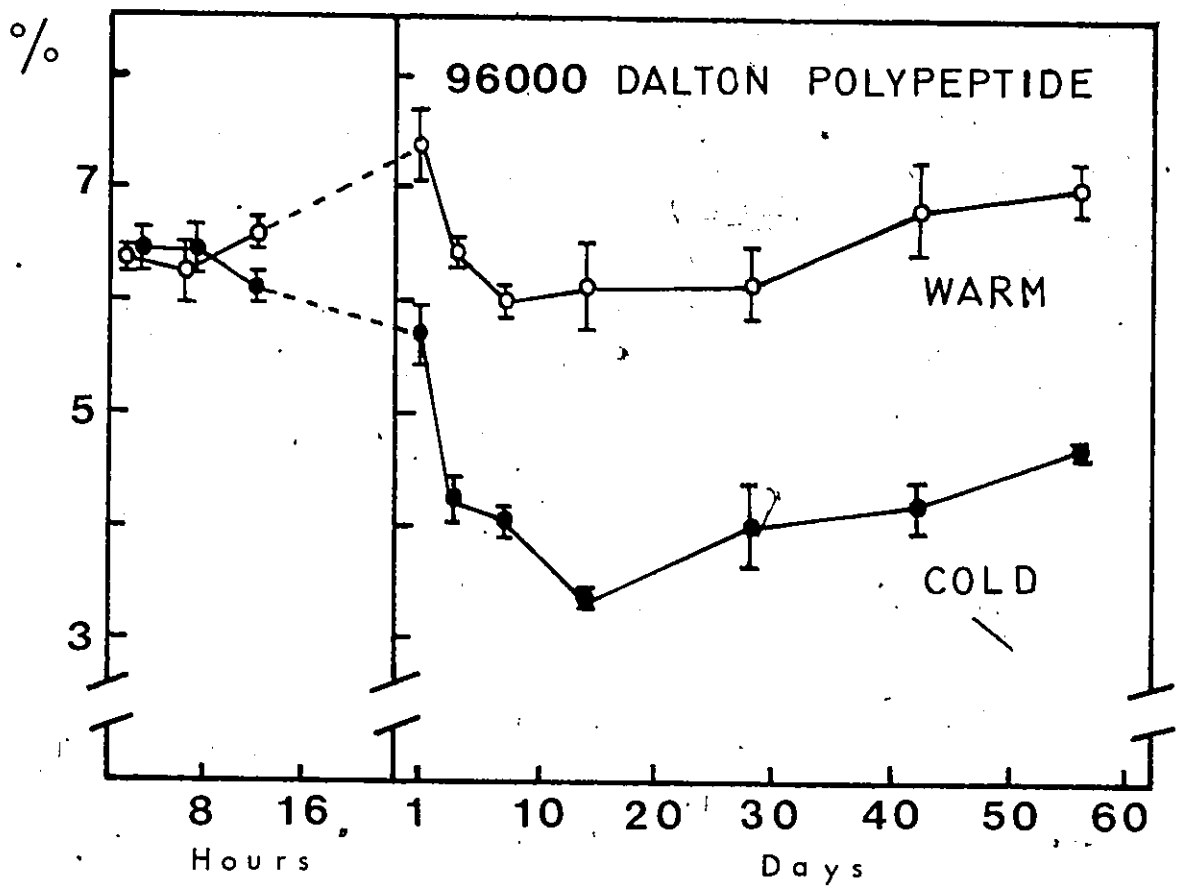

FIG. 29. Proportion of the 96 000 M.W. polypeptide of brown adipose tissue mitochondrial membrane proteins during acclimation of rats to cold.

Legend of this figure is the same as under Fig. 28.

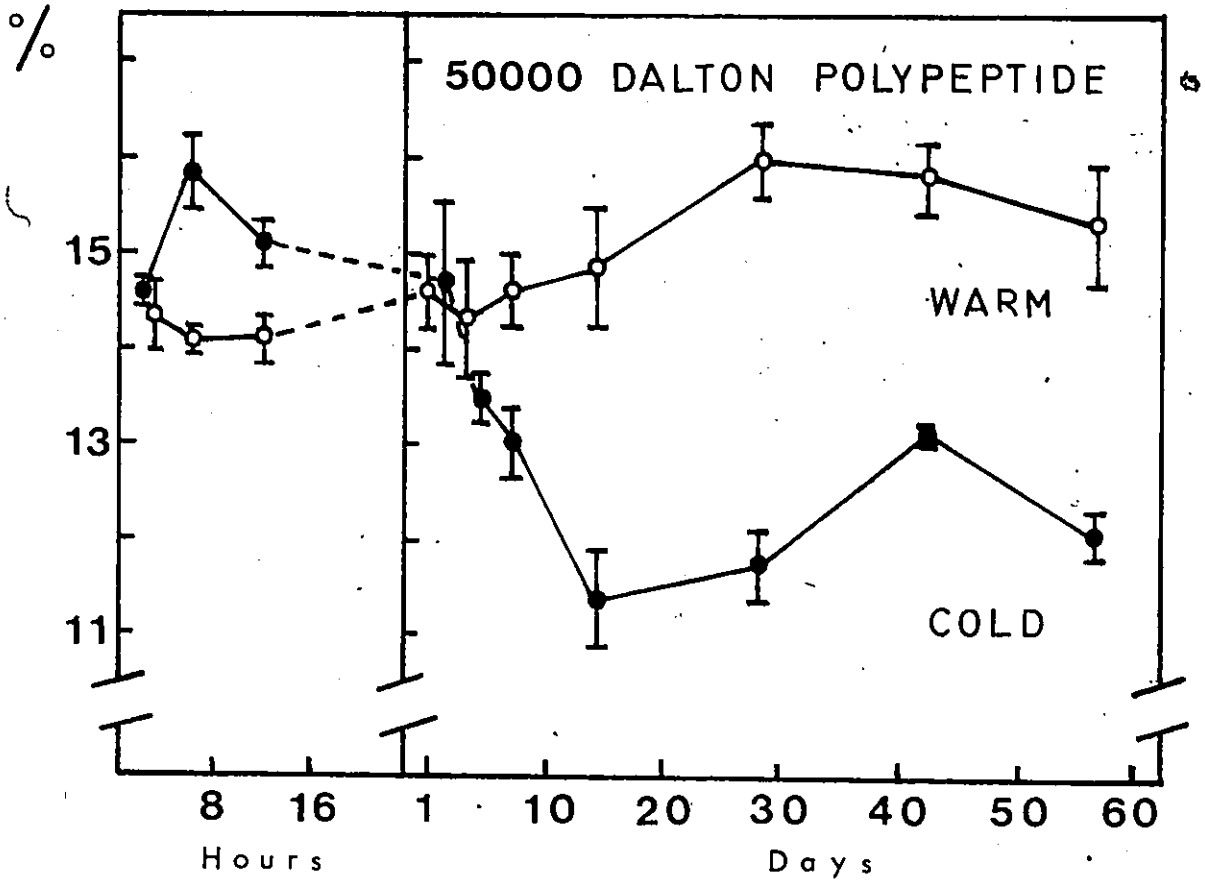

FIG. 30. Proportion of the 50 000 M.W. polypeptide of brown adipose tissue mitochondrial membrane proteins during acclimation of rats to cold.

Legend of this figure is the same as under Fig. 28.

Conclusions: The initial response to cold stress appears to involve unmasking of mitochondrial proton conductance pathways as suggested by the rapid increase in purine nucleotide binding to BAT mitochondria which precedes the increase in 32 000 polypeptide. This acute response to cold exposure is followed by a slower adaptive response characterized by the formation of new binding sites and a subsequent increase in concentration of pathways. Most probably, both the increase in BAT mass and the increase in concentration of mitochondrial proton conductance pathways brought by cold acclimation contribute to the enhanced capacity of the rats for nonshivering thermogenesis, known to increase progressively during the first three weeks of adaptation to cold (124).

2. Time-course of the appearance of changes in rat BAT mitochondrial structure during acclimation to cold

The structure of BAT mitochondria from C-A rats was found to be different from that of mitochondria from W-A rats (Part I, section B). Mitochondria from C-A rats are larger and have an altered internal structure. They have an increased surface area of inner membranes resulting from an increased concentration and a more organized arrangement of their cristae. It is important to establish whether these changes in mitochondrial structure are correlated with the changes in composition and also contribute to the development of nonshivering thermogenesis in the cold-exposed rat. In addition, changes in mitochondrial composition appear biphasic. There is a rapid unmasking of proton conductance pathways in the acute phase of cold exposure followed by a slower increase

in the concentration of the pathways when cold exposure is prolonged over several days. It is of interest to know whether these changes have a structural basis.

Objective: The purpose of this work is to find out whether the changes in mitochondrial structure observed are related to the development of cold adaptation or whether they are secondary to acute changes occurring in response to cold stress. The possibility that a reorganization of mitochondrial membranes could explain the unmasking of purine nucleotide binding sites in the acute phase of cold exposure is investigated.

Method: The experiment is performed exactly as described in the preceding method section (Part II, section A-1). The same mitochondrial preparations were used for both electron microscopy and size determination. To express the size distribution time-course in a quantitative way, the ratio of the total volume contributed by mitochondria of size greater than 0.5 μm^3 to the total volume contributed by mitochondria of size smaller than 0.5 μm^3 has been calculated. Mitochondria from warm-acclimated rats have a ratio of about 1.0. Ratios higher than 1.0 during acclimation to cold indicate that mitochondria are getting larger relative to these of warm-acclimated rats.

Results and discussion: Time course of the change in mitochondrial size distribution, as measured with the Coulter Counter, is illustrated in Fig. 31. The change is biphasic. Mitochondria are larger after the first hour of cold exposure, return to normal size by 24 hours and increase in volume again to reach a maximum ratio within a week.

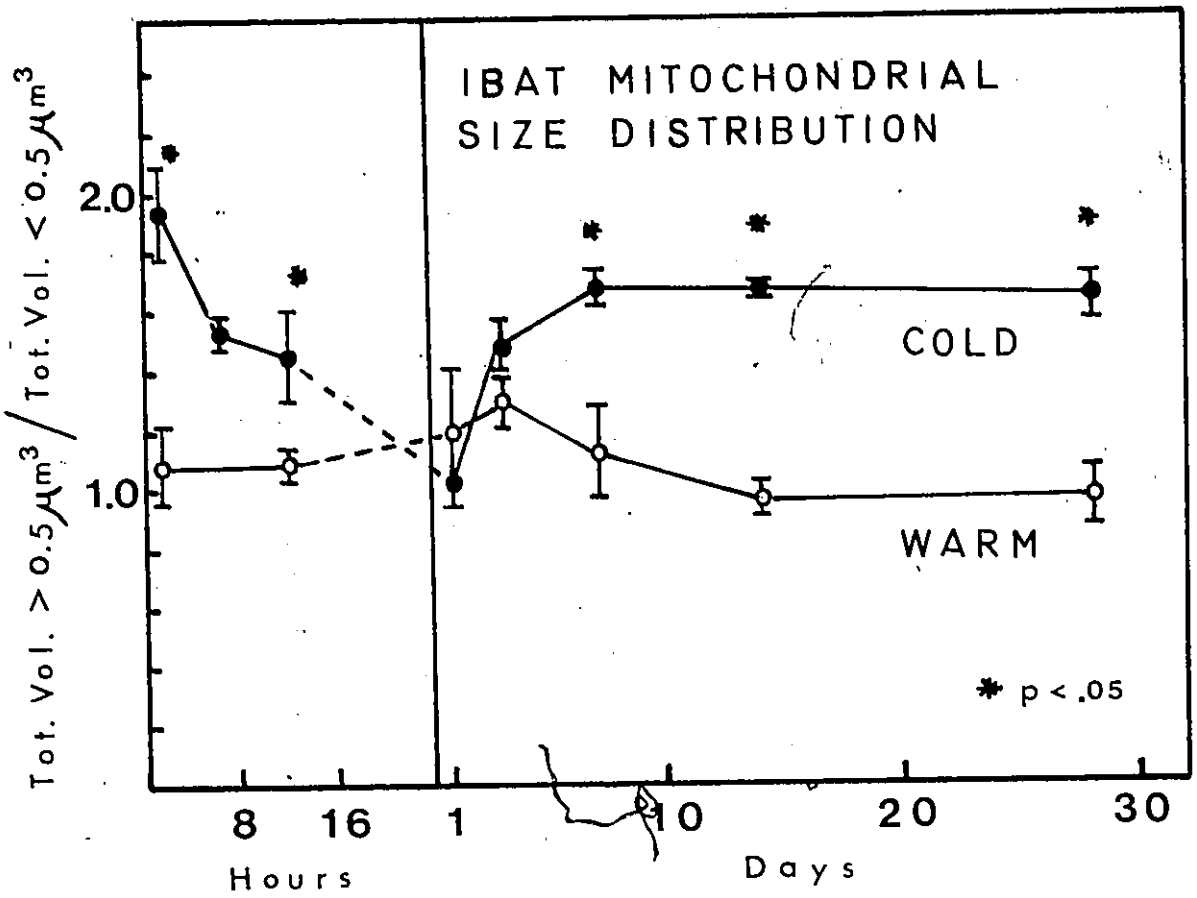

FIG. 31. Size distribution of brown adipose tissue mitochondria of rats during acclimation to cold.

Rats were kept at either 4°C (COLD) or 28°C (WARM) for the times indicated. The results are expressed as the ratio of the total volume contributed by mitochondria of size greater than 0.5 μm^3 to the total volume contributed by mitochondria of size smaller than 0.5 μm^3. The values are the means ± S.E. of 3 experiments.

Changes in mitochondrial internal structure are also evident from the electron micrographs presented in Fig. 32 - 45. A biphasic transformation is also observed. Within the first hour of exposure to cold, a rearrangement of mitochondrial inner membranes appears to occur. The "dotted" appearance of mitochondria from W-A rats undergoes a transformation to a more parallel arrangement of the cristae (compare Fig. 32 and 33). This difference however vanishes within 24 hours (Fig. 34 - 37). After three days of exposure to cold, the number of mitochondria with parallel cristae increases again and a certain number of mitochondria with odd internal structure may be observed (Fig. 38). After two weeks spent in the cold, mitochondria with "dotted" internal structure have completely disappeared (Fig. 42). Mitochondria with a large number of parallel cristae are characteristic of C-A rats and maintain this type of internal structure as long as the rats live in the cold (4 weeks W-A and C-A, Fig. 43, 44; 9 weeks C-A and W-A, Fig. 55, 56). Such an increase in the number of cristae per mitochondria has previously been reported in tissue sections from BAT of cold-acclimated rats (271).

There is then a correlation between the time-course of changes in BAT mitochondrial composition and the time-course of changes in BAT mitochondrial structure during the first hours of cold exposure. It appears possible that the initial unmasking of purine nucleotide binding sites may be the result of mitochondrial membrane reorganization. Similar changes in mitochondrial structure have been shown in tissue sections from rats acutely exposed to cold. It was shown that within 24 hours of cold exposure, mitochondria of BAT vary greatly in size mainly due to an increase in size of the larger mitochondria (240). A swollen appearance of mitochondria in tissue sections from warm-acclimated or newborn rats

FIG. 32. IBAT mitochondria isolated from rats exposed to warm (28°C) for 1 hour. (x 16 000)

FIG 33. IBAT mitochondria isolated from rats exposed to cold (4°C) for 1 hour. (x 16 000)

32

33

FIG. 34. IBAT mitochondria isolated from rats exposed to warm (28°C) for 6 hours. (x 16 000)

FIG. 35. IBAT mitochondria isolated from rats exposed to cold (4°C) for 6 hours. (x 16 000)

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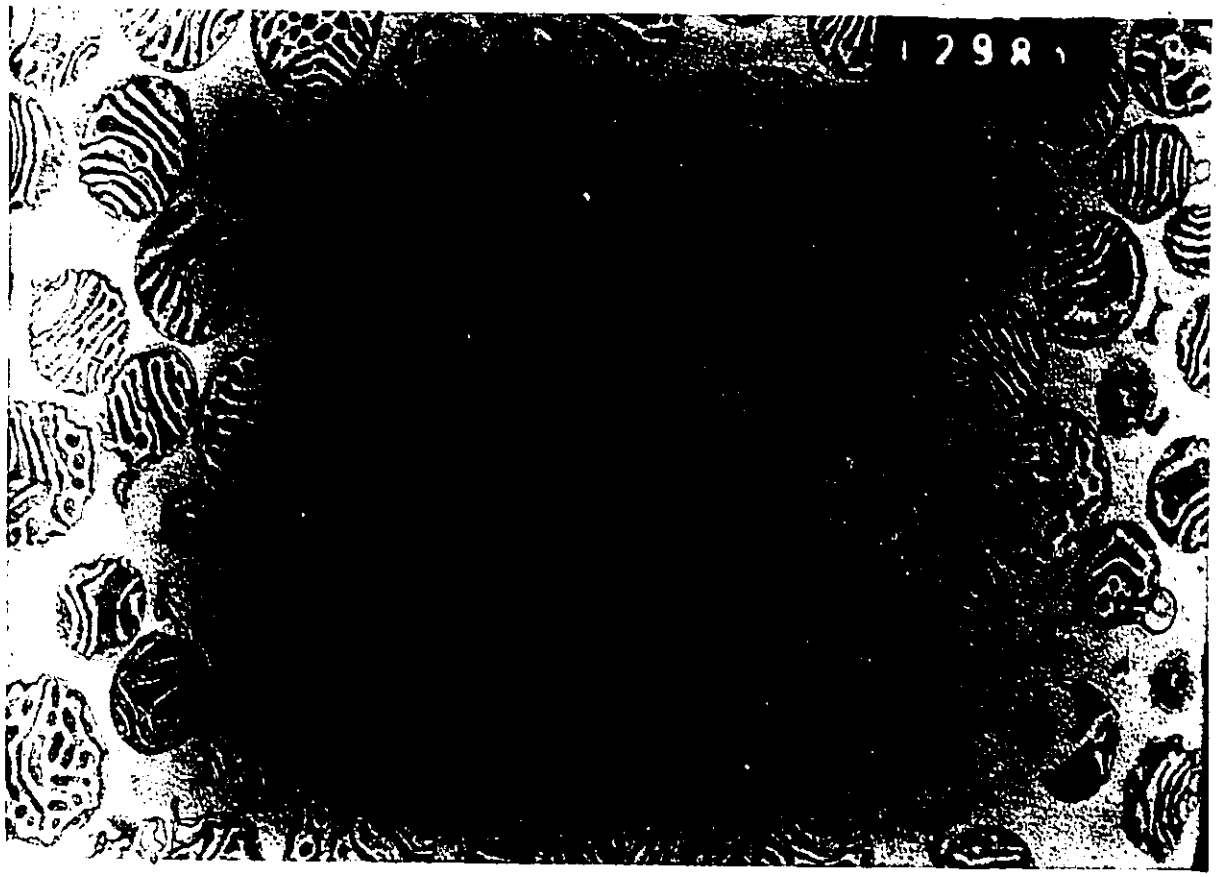

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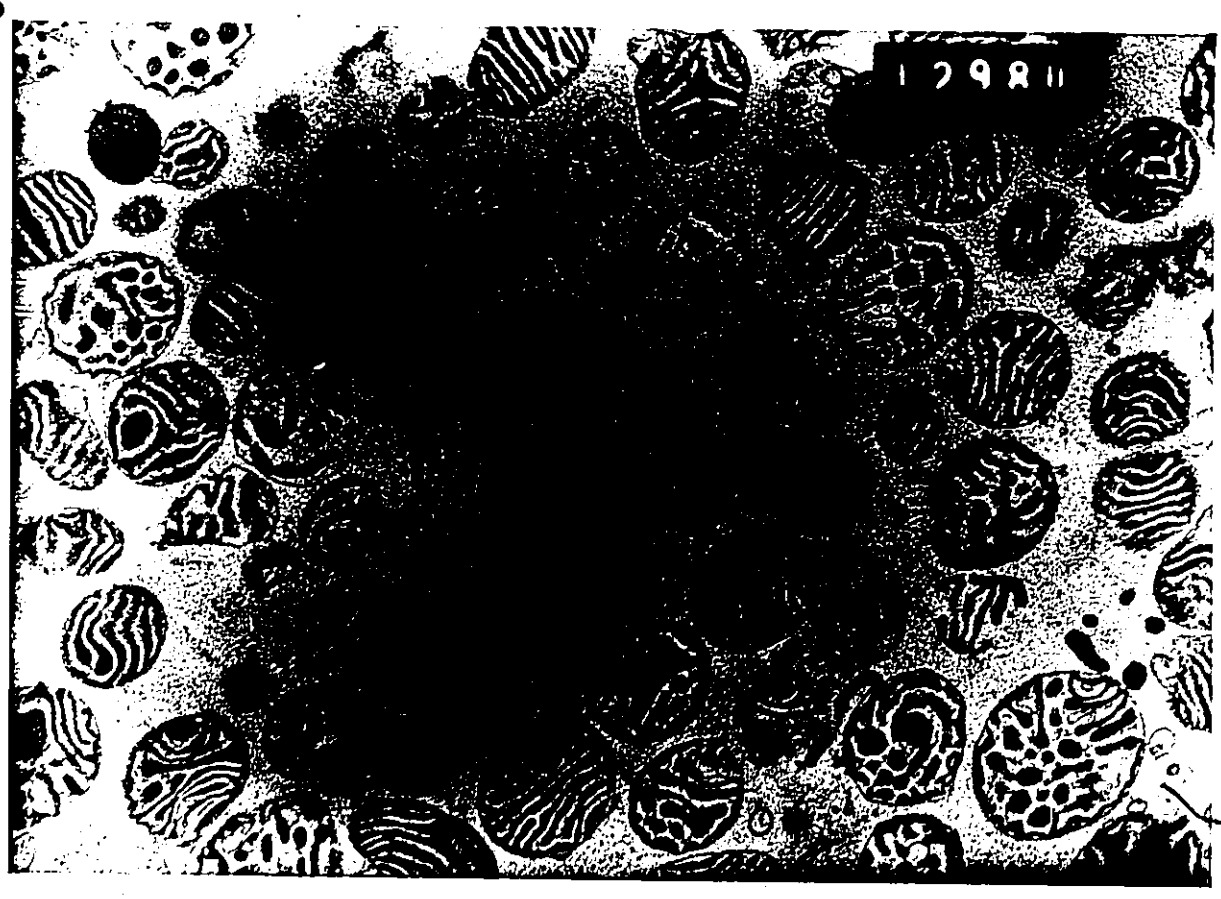
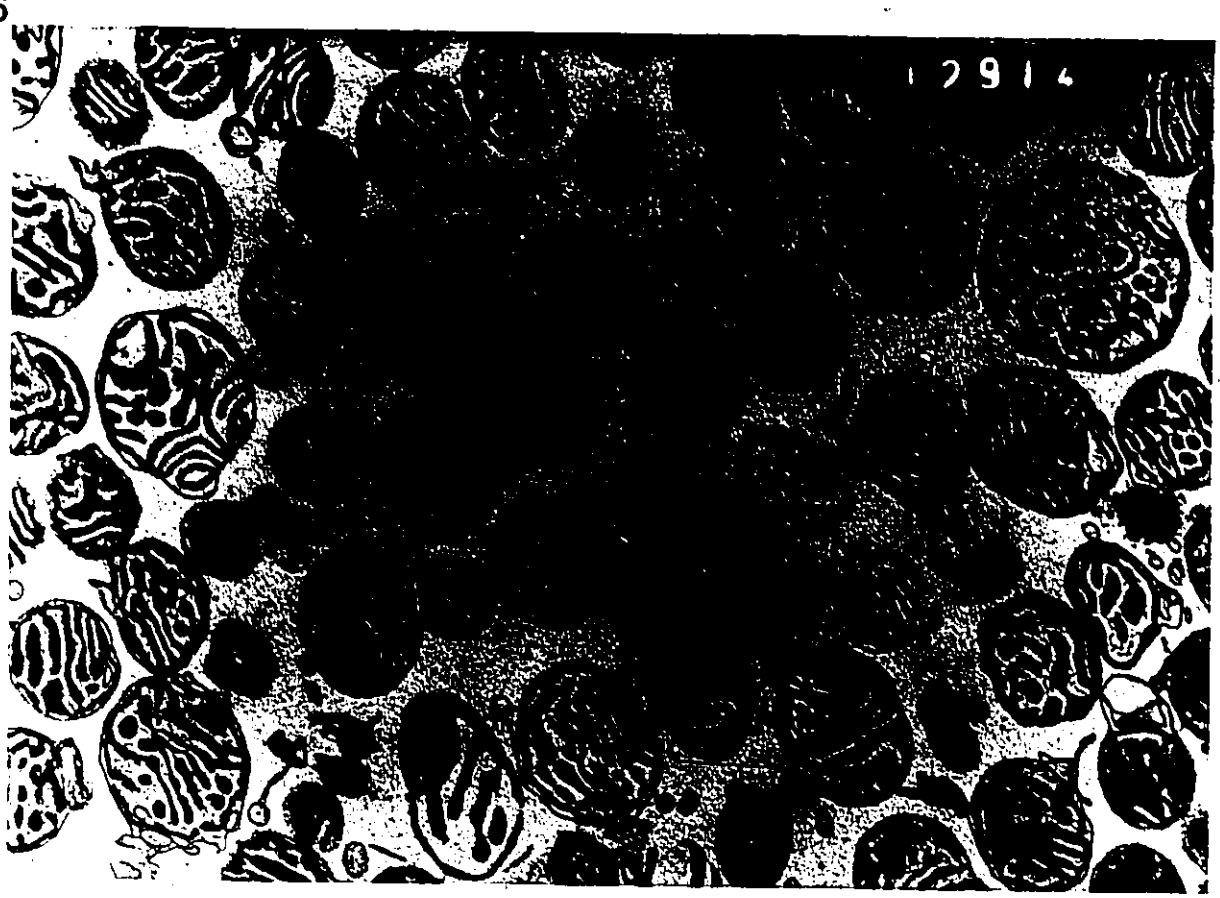


FIG. 36. IBAT mitochondria isolated from rats exposed to cold (4°C) for 12 hours. (x 16 000)

FIG. 37. IBAT mitochondria isolated from rats exposed to cold (4°C) for 1 day. (x 16 000)

36



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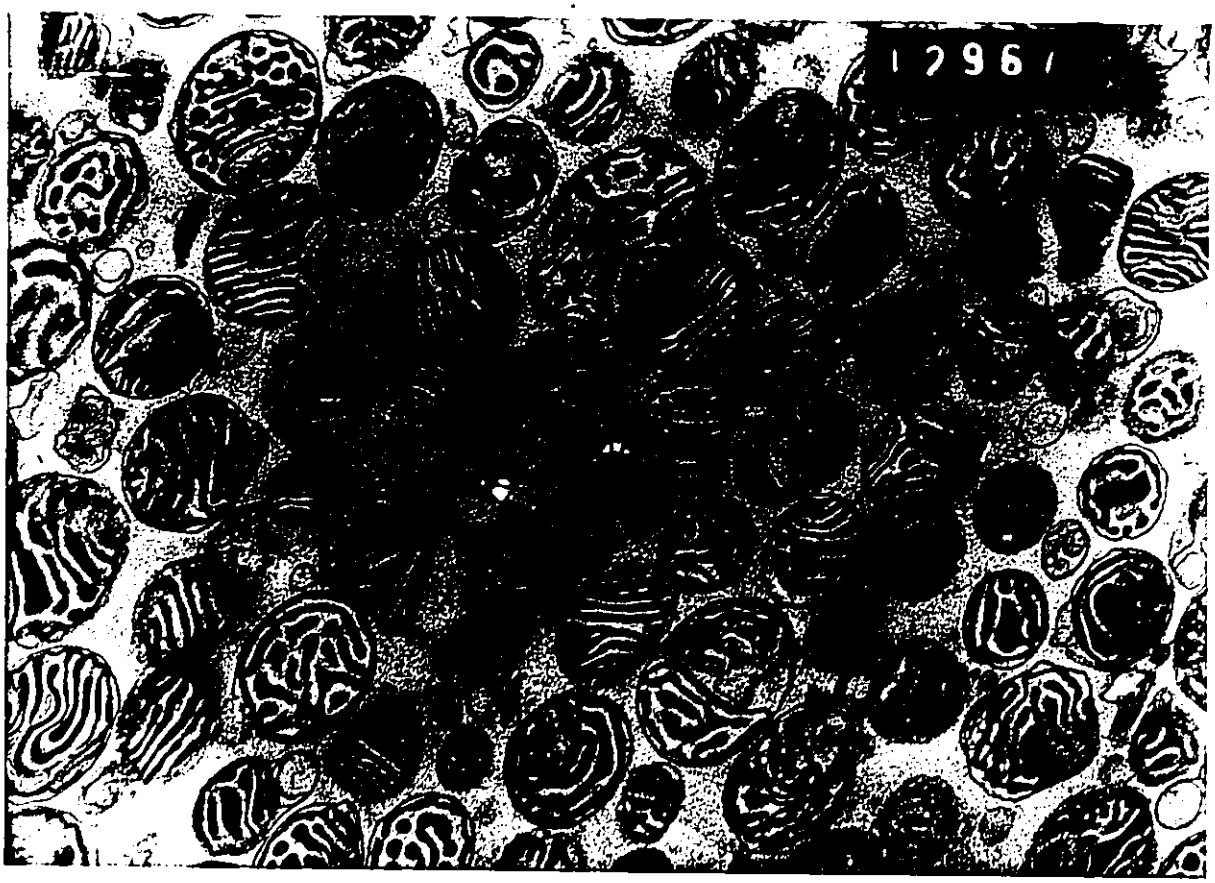

FIG. 38. IBAT mitochondria isolated from rats exposed to cold (4°C) for 3 days. (x 16 000)

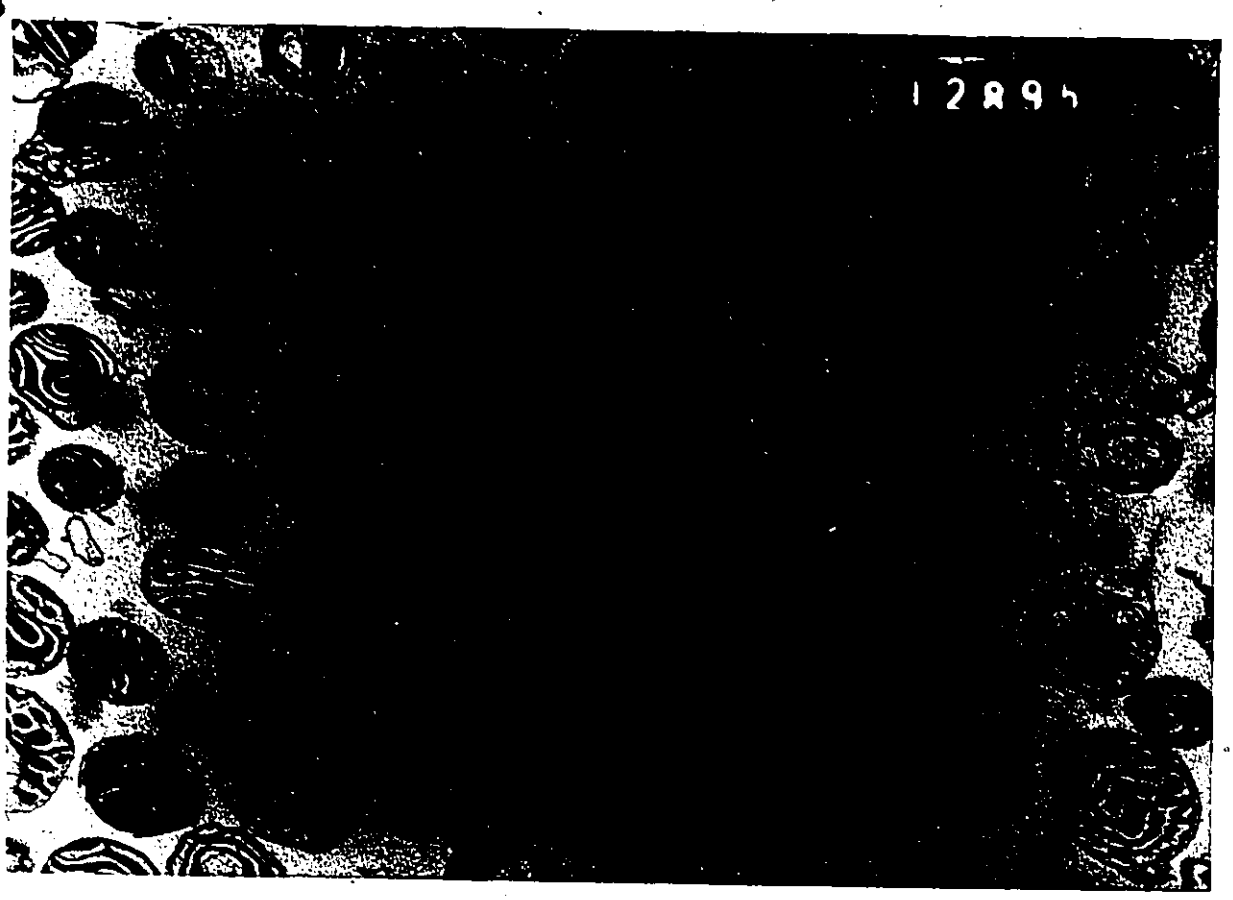
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FIG. 39. IBAT mitochondria isolated from rats exposed to warm (28°C) for 7 days. (x 16 000)

FIG. 40. IBAT mitochondria isolated from rats exposed to cold (4°C) for 7 days. (x 16 000)

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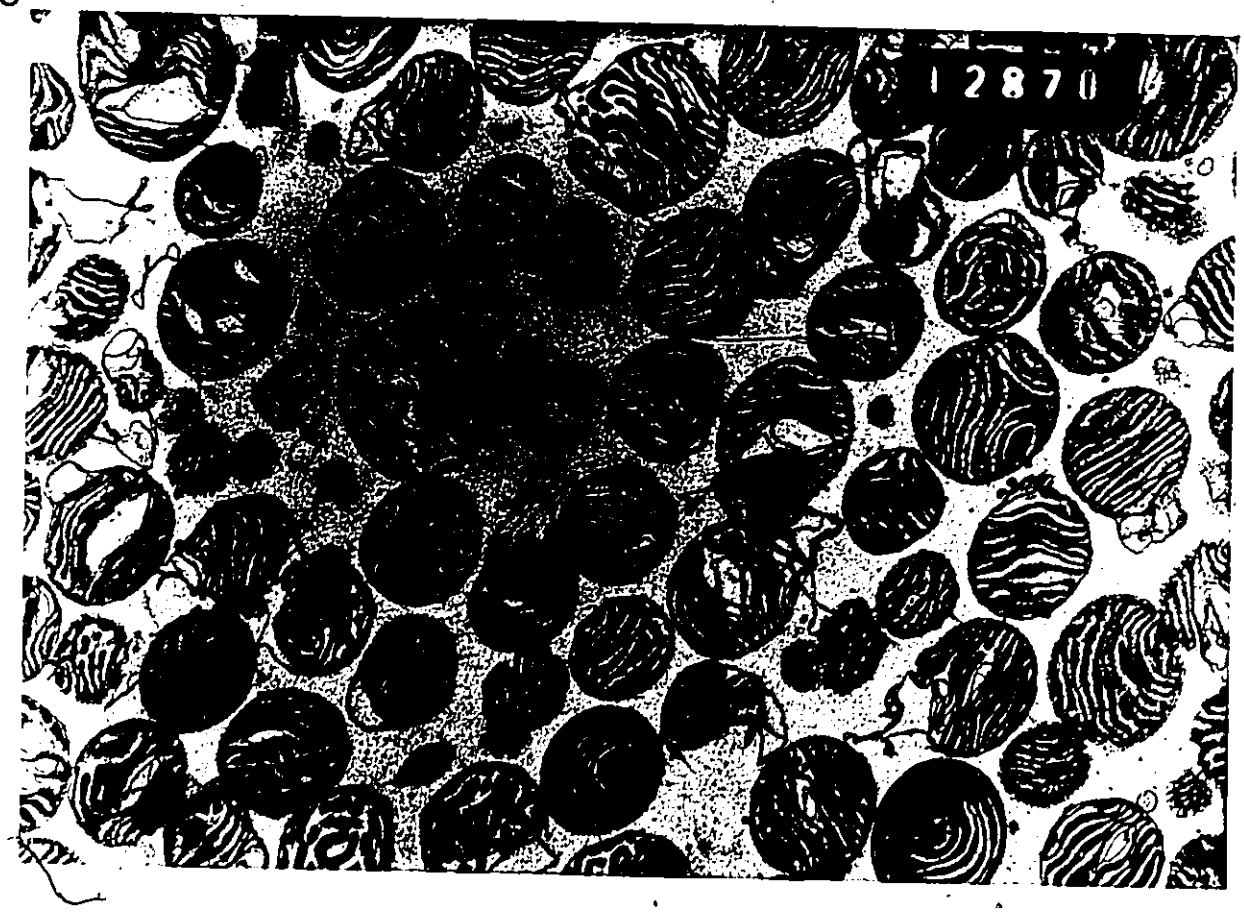

FIG. 41. IBAT mitochondria isolated from rats exposed to warm (28°C) for 14 days. (x 16 000)

FIG. 42. IBAT mitochondria isolated from rats exposed to cold (4°C) for 14 days. (x 16 000)

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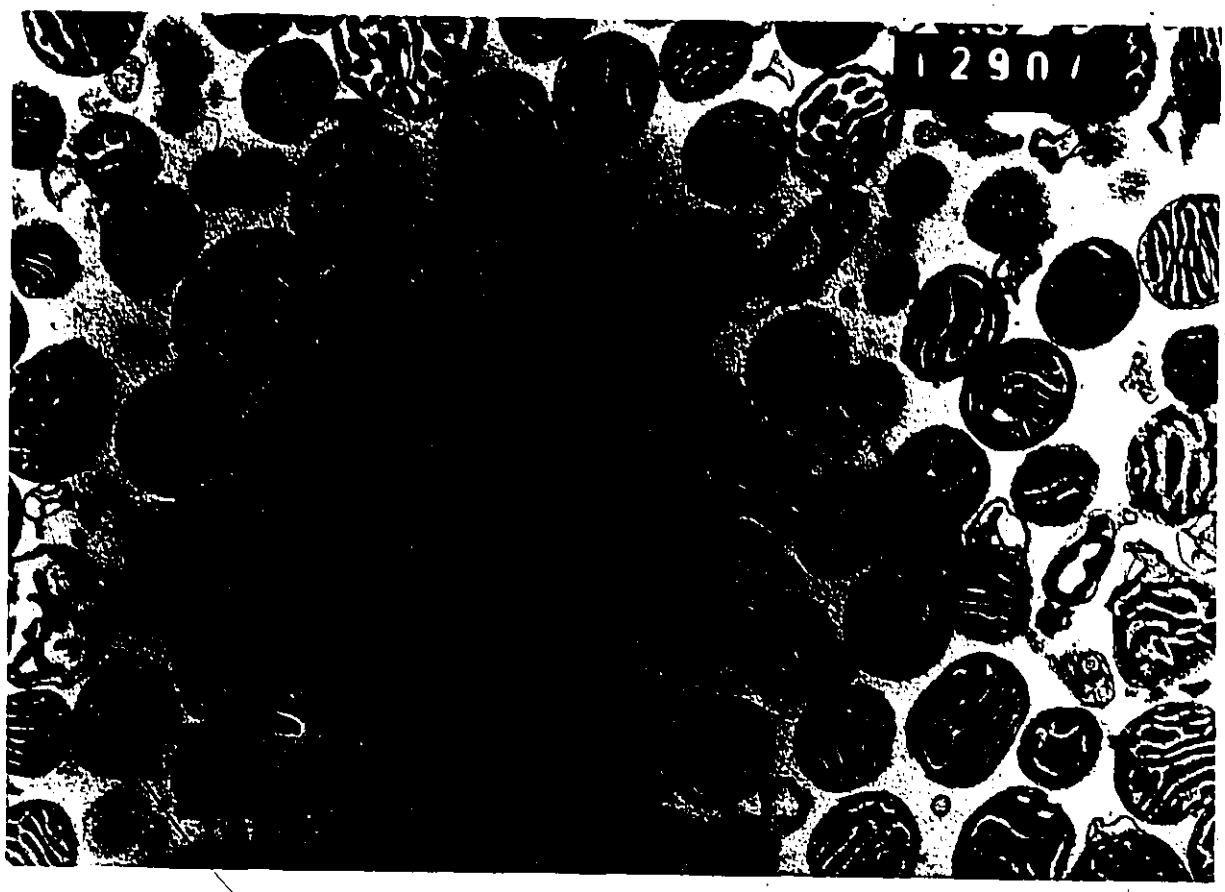

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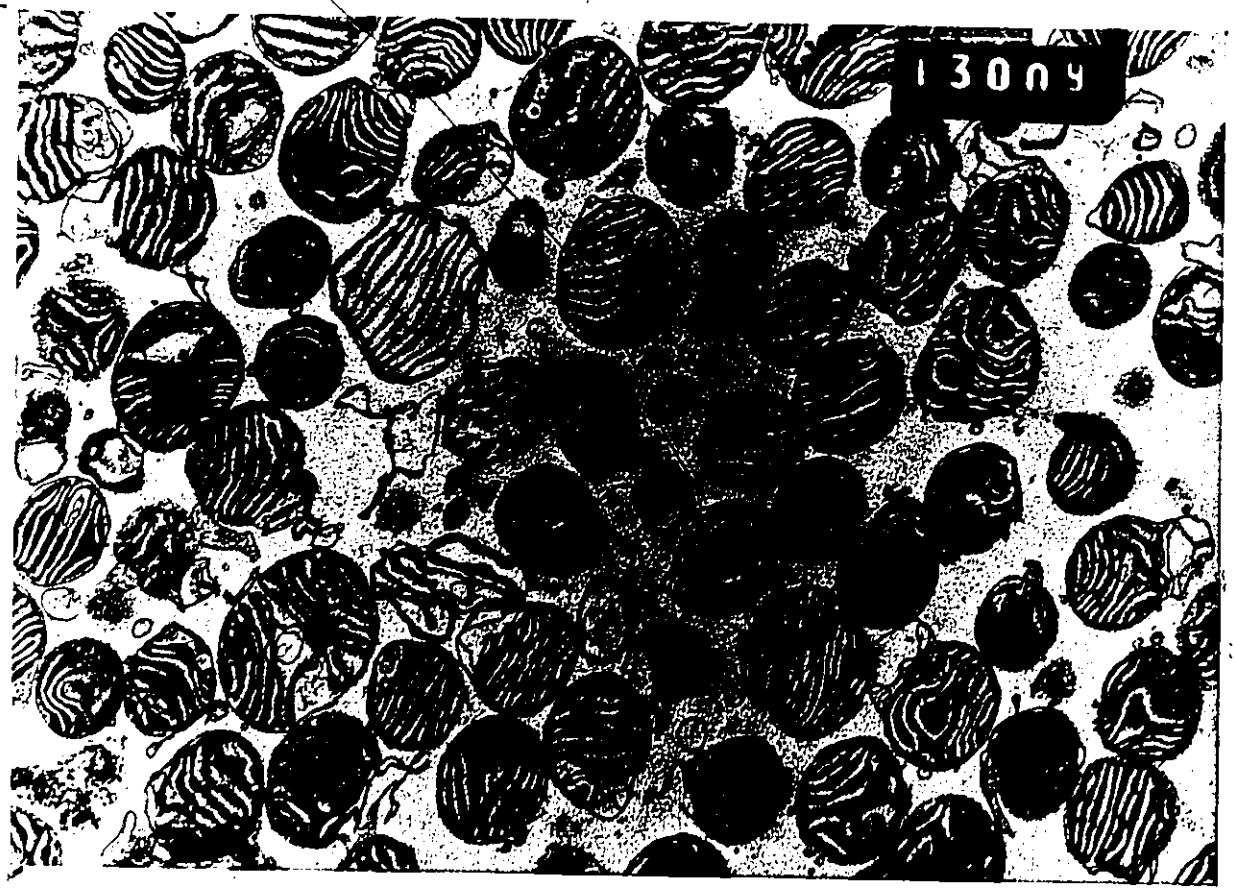
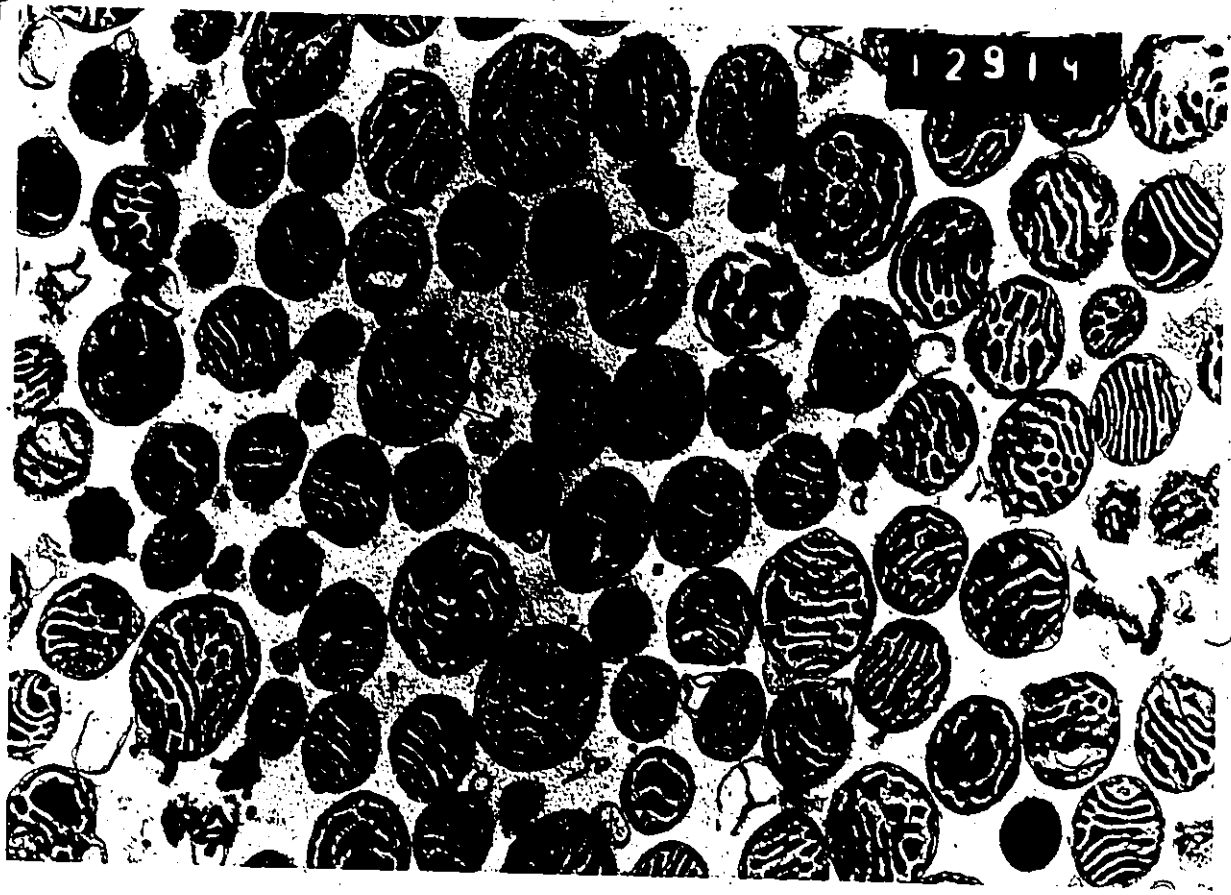


FIG. 43. IBAT mitochondria isolated from rats exposed to warm (28°C) for 28 days. (x 16 000)

FIG. 44. IBAT mitochondria isolated from rats exposed to cold (4°C) for 28 days. (x 16 000)

43



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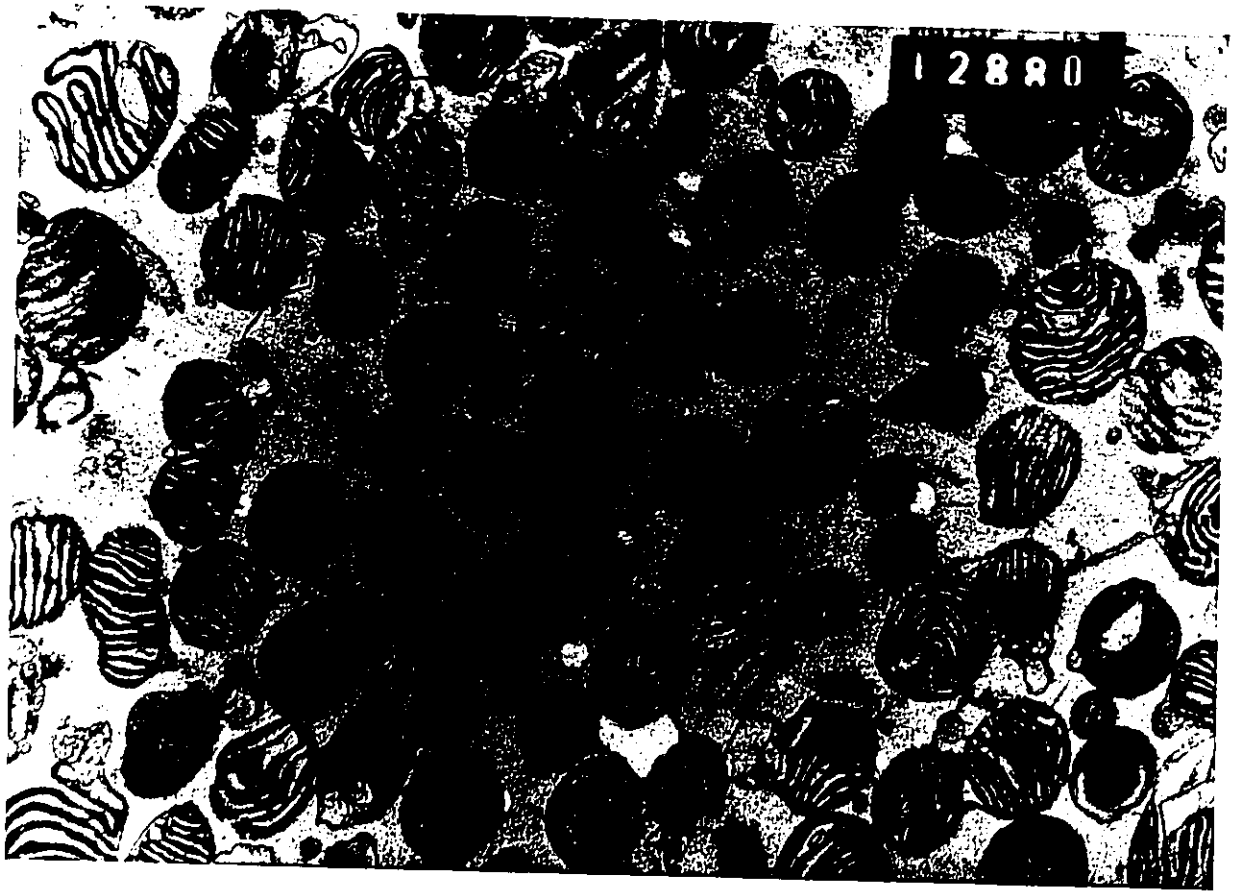


acutely exposed to cold was also reported (270, 279). However, it is important to note that the increase in purine nucleotide binding may not be directly linked to changes in mitochondrial internal structure. It is difficult to establish a definitive cause-effect relationship from the observations reported here. Several hypotheses may be considered. For example, it is possible that the tissue ion redistribution caused by NE upon acute cold exposure induces a change in mitochondrial internal structure and is responsible for the exposition of binding sites which were previously unavailable for binding. But it is as likely an explanation that the exposition or activation of purine nucleotide binding sites by NE by some unknown mechanisms result in an altered ion distribution and osmotic properties of the mitochondria and give rise to the altered morphology observed on the electron micrographs. The term "unmasking" will thus be used in this thesis in a general sense meaning the new availability of sites for binding without any de novo synthesis (see Section III-A) and without reference to any mechanism by which such change may occur.

On prolonged exposure to cold, there is a greater abundance and parallelism of cristae within the mitochondria. However, there is an overlap between changes in internal mitochondrial structure at different exposure times. Mitochondria do not seem to be all altered at the same time. This can be observed in Fig. 38 and 40 where some mitochondria with "dotted" internal structure can still be observed among the more numerous mitochondria with parallel cristae. This may be due to individual differences in the rate of response of mitochondria or to the age distribution of the mitochondrial population. The half-life of mitochondrial proteins of BAT is about 10-12 days in W-A rats and 7-9 days in C-A rats (26). The large mitochondrial proliferation known to occur between three

and fourteen days of exposure to cold (279) most probably results in a large alteration of the age distribution of the mitochondrial population within the tissue.

Conclusions: The transformation of mitochondrial structure is biphasic. A reorganization of mitochondrial membranes occurs in the acute phase of cold acclimation that might be responsible for the unmasking of purine nucleotide binding sites also occurring in this period.

On prolonged cold exposure, simultaneously with the increase in concentration of proton conductance pathways, there is also an increase in the amount of inner membrane material of BAT mitochondria. This increased amount of inner membrane material most certainly provides support not only for the increased proportion of proton channels, but also for the various substrate dehydrogenases and other enzymes known to increase in BAT mitochondria upon cold acclimation (254, 280).

B. DEACCLIMATION TO COLD

1. Time-course of the disappearance during deacclimation of the differences in BAT mitochondrial composition of C-A rats

Nonshivering thermogenesis in the rat is an adaptive process, being only present in the adult to any appreciable extent after adaptation to living in the cold and disappearing when the animal is returned to normal temperature (124). It was shown in the previous section that BAT mass increases during adaptation to cold. The changes in mitochondrial composition are biphasic. The acute phase of cold acclimation is charac-

terized by an unmasking of purine nucleotide binding sites while the adaptive phase is characterized by a progressive increase in the concentration of proton conductance pathways as measured by the increase in 32 000 polypeptide. If the changes observed in mitochondrial composition in the adaptive phase are related to the thermogenic state of the tissue, they should disappear when the C-A rats are returned to warm temperature.

Objective: The purpose of this work is to find out whether the changes in mitochondrial composition observed in C-A rats are related to the thermogenic state of the tissue (i.e. whether they regress when the C-A rats are returned to warm room) and whether this regression is also biphasic.

Method: A large group of rats that had been acclimated to cold for 5 weeks were separated into 2 groups and either left in the cold room or returned to the warm room for the following times: 1, 3, 7, 14, 28 and 42 days. The same measurements as in the previous section were made on 3 cold-acclimated rats left at 4°C and 3 cold-acclimated rats that had been returned to 28°C after each of the time indicated. Purine nucleotide binding and peptide pattern were obtained on the same mitochondrial preparations.

Results and discussion: The brown fat mass of C-A rats returned to 28°C decreases very rapidly. The weight wet of the tissue reaches a minimum value within 7 days of readaptation to warm (Fig. 45). The time course of the decrease in protein content follows the same pattern

(Fig. 46). Binding of GDP (Fig. 47), of ADP (Fig. 48) and the proportion of 32 000 polypeptide (Fig. 49) show a maximum rate of decrease within 3-7 days after removal from the cold followed by a much slower disappearance thereafter. The regression of all the cold-induced changes in mitochondrial composition follows then a similar time-course, being almost completed within the first 7 days of readaptation to warm.

A similar regression rate has been observed for the enhanced metabolic response to catecholamines after removal of C-A rats from the cold (157). The rate of regression appears linked to the readaptation temperature. The rate of regression of the changes in BAT observed in this thesis where the C-A animals are returned to a warm room at 28°C is faster than those observed at 25°C (157) or 22°C (271). High regression rate is however not specific to BAT. Cold-acclimated rats are known to have a greater increase in heart rate in response to subcutaneous administration of isoproterenol (185). By 4 days after removal from the cold and transfer to 25°C, the responsiveness of heart rate to isoproterenol in the cold-treated group no longer differs from that of the warm-acclimated group (185).

It is also known that the turnover of total mitochondrial proteins in BAT is increased in C-A rats (26). The half-life of mitochondrial protein is about 7 days in C-A rats and 11 days in W-A rats (26). Mitochondrial protein synthesis is required for the proliferation of BAT mitochondria and for the maintenance of the altered mitochondria in the cold-acclimated state (280). The BAT content of cytochrome oxidase of 2 weeks cold-exposed rats returns to basal values within 7 days when mitochondrial protein synthesis is blocked by oxytetracycline (130). It appears then possible that an inhibition of protein synthesis in the BAT

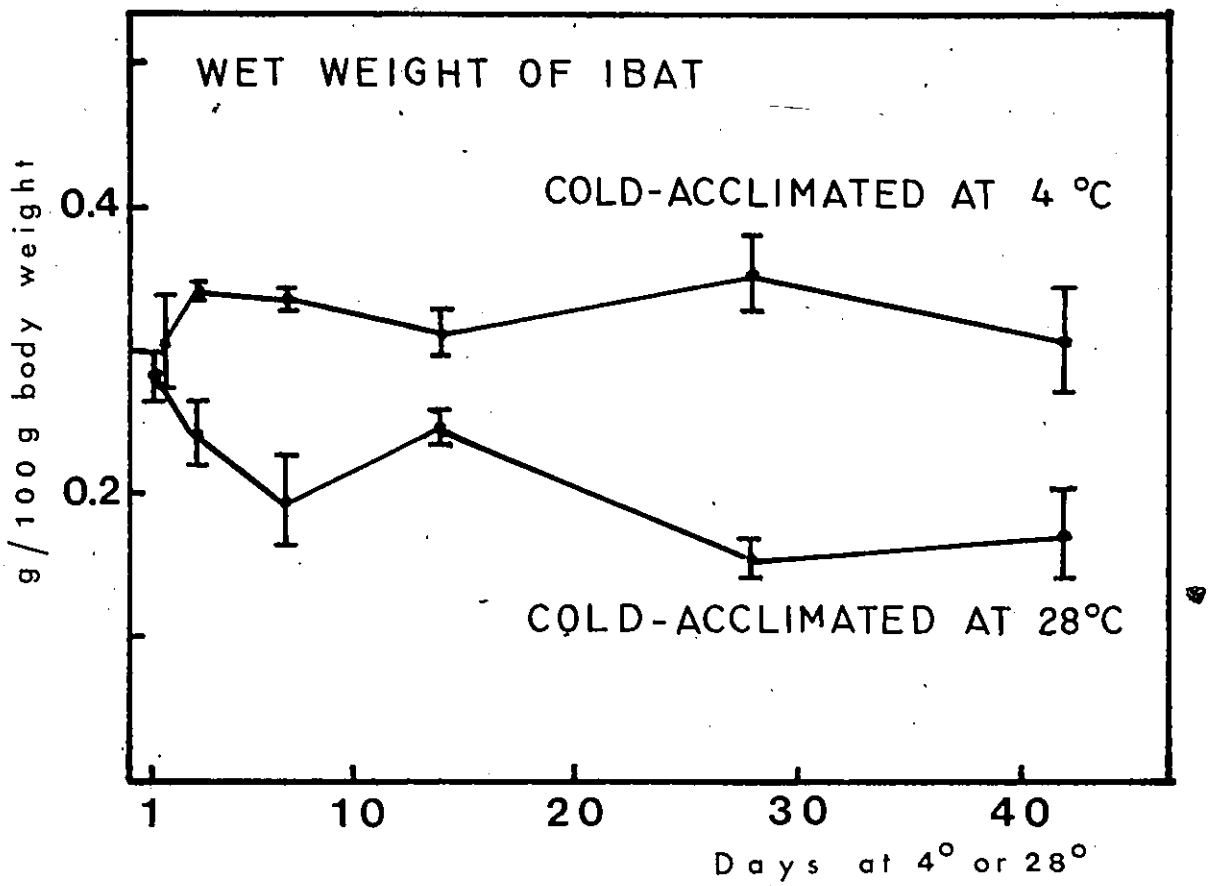


FIG. 45. Regression of interscapular brown adipose tissue during readaptation of cold-acclimated rats to warm (28°C).

Rats that had been cold-acclimated for five weeks are either kept at 4°C (C-A AT 4°C) or returned to the warm room (C-A AT 28°C) for the times indicated. Values are the means ± S.E. of 3 observations.

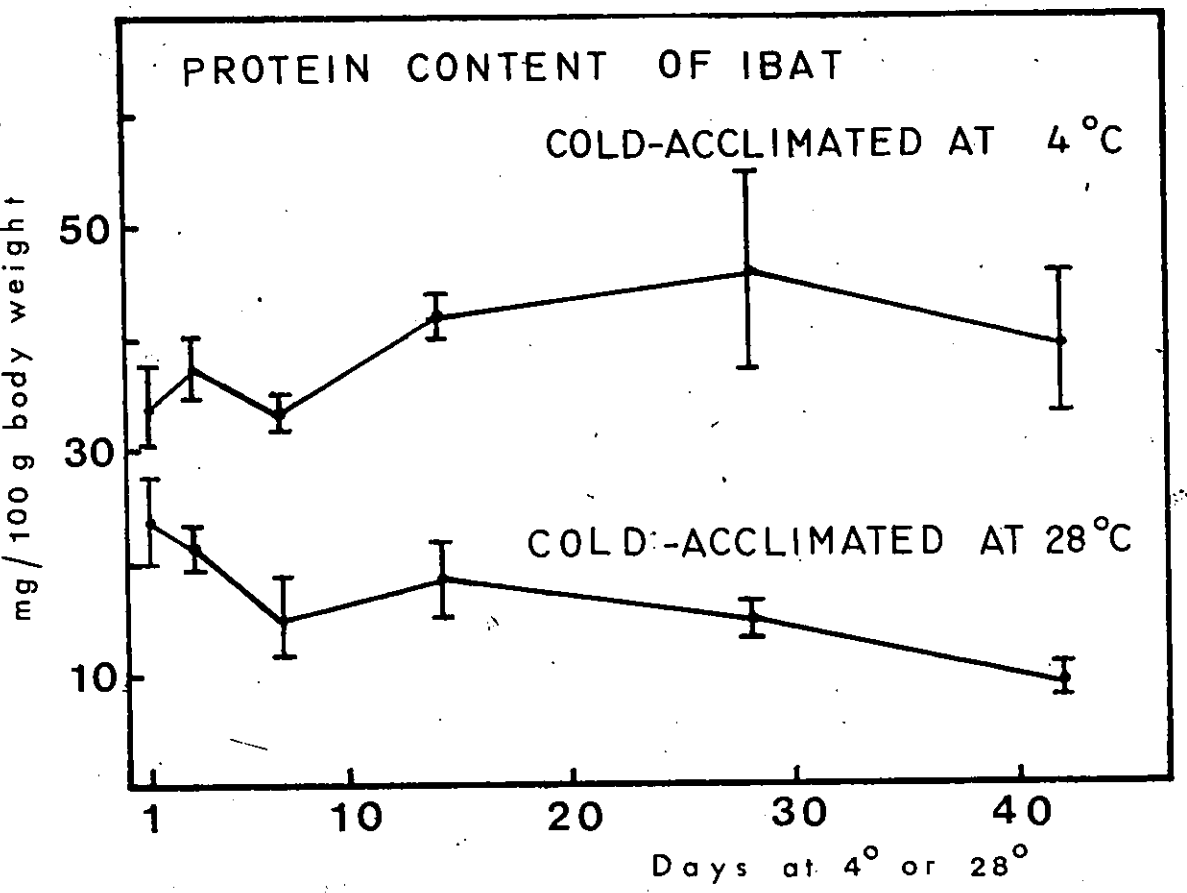


FIG. 46. Protein content of interscapular brown adipose tissue during readaptation of cold-acclimated rats to warm.

Rats that had been cold-acclimated for 5 weeks are either kept at 4°C (C-A AT 4°C) or returned to the warm room (C-A AT 28°C) for the times indicated. Values are the means ± S.E. of 3 observations.

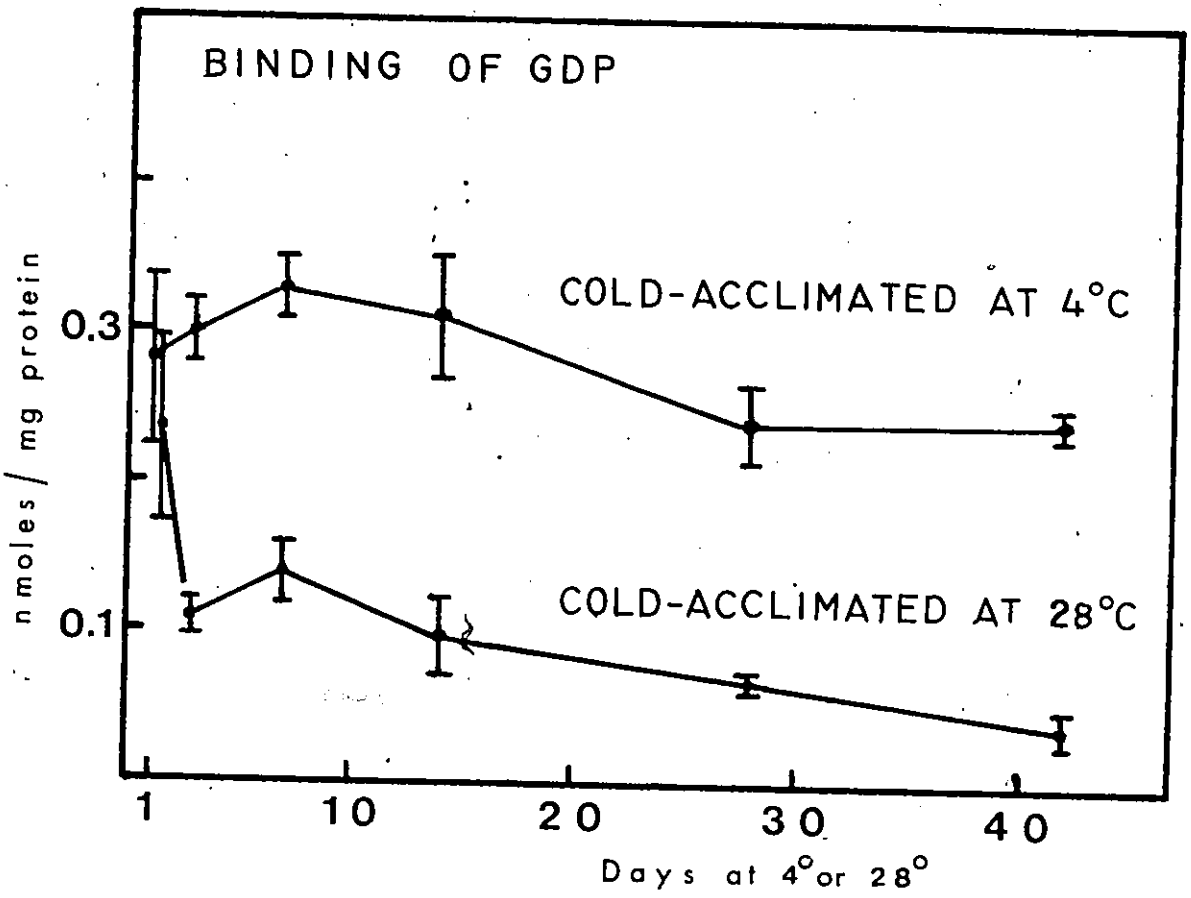


FIG. 47. Binding of GDP to BAT mitochondria during readaptation of cold-acclimated rats to warm.

Rats that had been cold-acclimated for 5 weeks are either kept at 4°C (C-A AT 4°C) or returned to the warm room (C-A AT 28°C) for the times indicated. Values are the means ± S.E. of 3 observations.

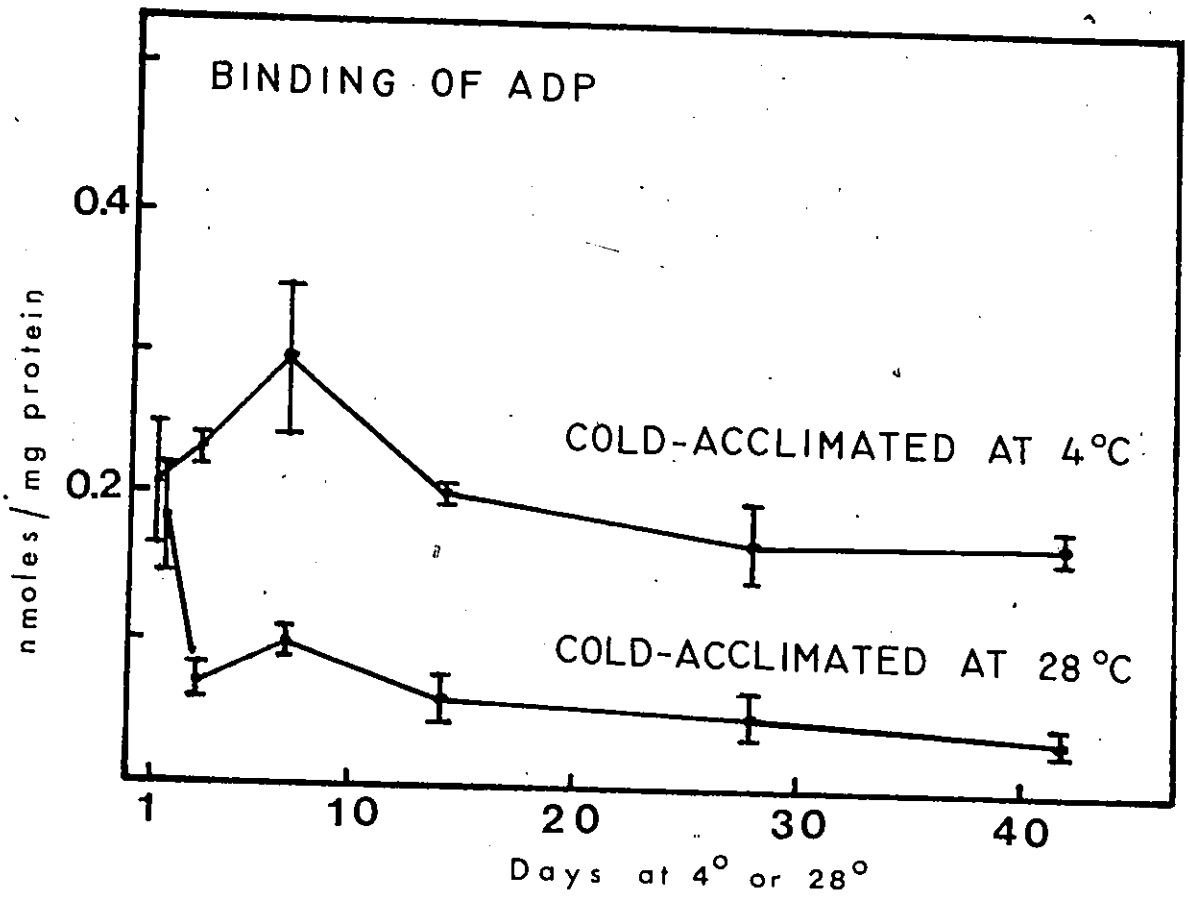


FIG. 48. Binding of ADP to brown adipose tissue mitochondria during readaptation of cold-acclimated rats to warm.

Legend is the same as under Fig. 47.

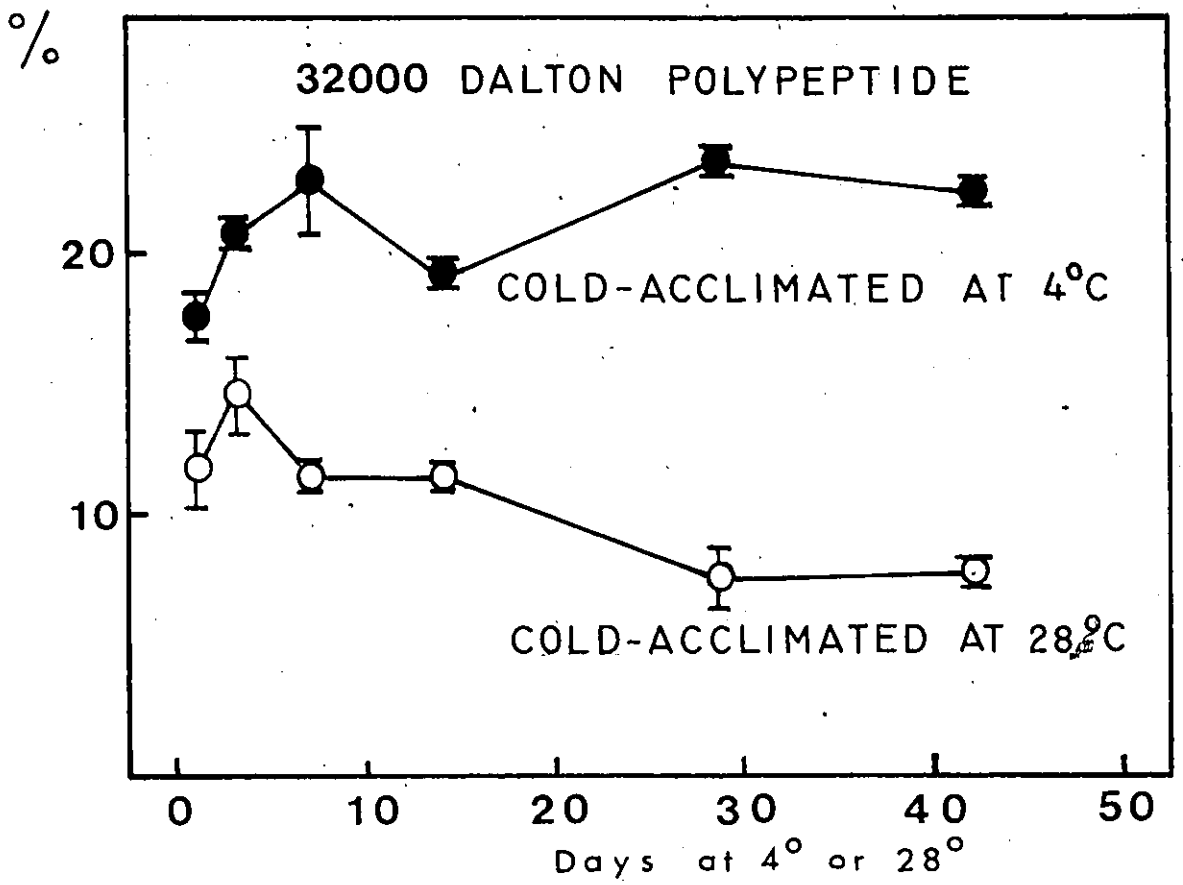


FIG. 49. Proportion of the 32 000 M.W. polypeptide of brown adipose tissue mitochondrial membrane proteins during readaptation of cold-acclimated rats to warm.

Five weeks cold-acclimated rats were either left in the cold (C-A AT 4°C) or returned to the warm room (C-A AT 28°C) for the times indicated. Values are the means ± S.E. of 3 observations.

of C-A rats known to have high mitochondrial protein turnover is responsible for the rapid regression observed when these animals are removed from the cold. Whether there is also an increased activity of cytoplasmic or lysosomal proteases occurring in the tissue upon deacclimation is unknown.

Conclusions: The concentration of proton conductance pathways decreases when the cold-acclimated animals are returned to normal temperature in parallel with the known thermogenic state of the tissue. It is then very likely to be a major determinant of nonshivering thermogenesis in BAT of the rat. The rate at which mitochondria are altered upon cold-acclimation is different from the rate at which the changes observed regress upon deacclimation. This will be discussed in more detail after the next section (Part II B, section 2).

2. Time-course of the disappearance during deacclimation of the differences in BAT mitochondrial structure of C-A rats

Structure of BAT mitochondria is altered upon cold-acclimation (see Part I, section B). The changes in mitochondrial structure are biphasic. There is a reorganization of mitochondrial inner membranes apparent during the first hour of exposure to cold followed by a progressive increase in the concentration and parallelism of the cristae. These changes are paralleled by changes in mitochondrial size. All the cold-induced changes in mitochondrial composition regress rapidly within 7 days after the removal of the C-A rats from the cold.

Objective: The purpose of this work is to find out whether changes in mitochondrial structures parallel the regression of the changes in mitochondrial composition.

Method: The experiment was performed exactly as described in the preceding section (Part II-B, section 1). The same mitochondrial preparations were used for both electron microscopy and size determination.

Results and discussion: Mitochondria from C-A rats decrease in volume upon readaptation to warm and the same size distribution as in the W-A rats is observed within 7 days (Fig. 50). Similarly, mitochondria with a "dotted" internal structure, non-existent in C-A rats, reappear within 7 days of readaptation to warm (Fig. 51-56). The disappearance of cold-induced changes in mitochondrial composition is then paralleled by the disappearance of cold-induced changes in mitochondrial structure upon readaptation of C-A rats to warm.

The disappearance of cold-induced changes in mitochondrial structure has previously been observed in tissue sections upon deacclimation (271).

The rate at which mitochondria are altered upon cold-acclimation is different from the rate at which the cold-induced changes regress upon deacclimation. Upon exposure to cold, BAT grows maximally during the first 3-7 days and at a slower rate for the next 20 days. The time-course of the appearance of changes in BAT mitochondrial structure and composition during acclimation to cold is biphasic. However, upon deacclimation, the brown fat mass regresses completely within 7 days and all the cold-induced mitochondrial changes in structure and composition disappear at the same rate.

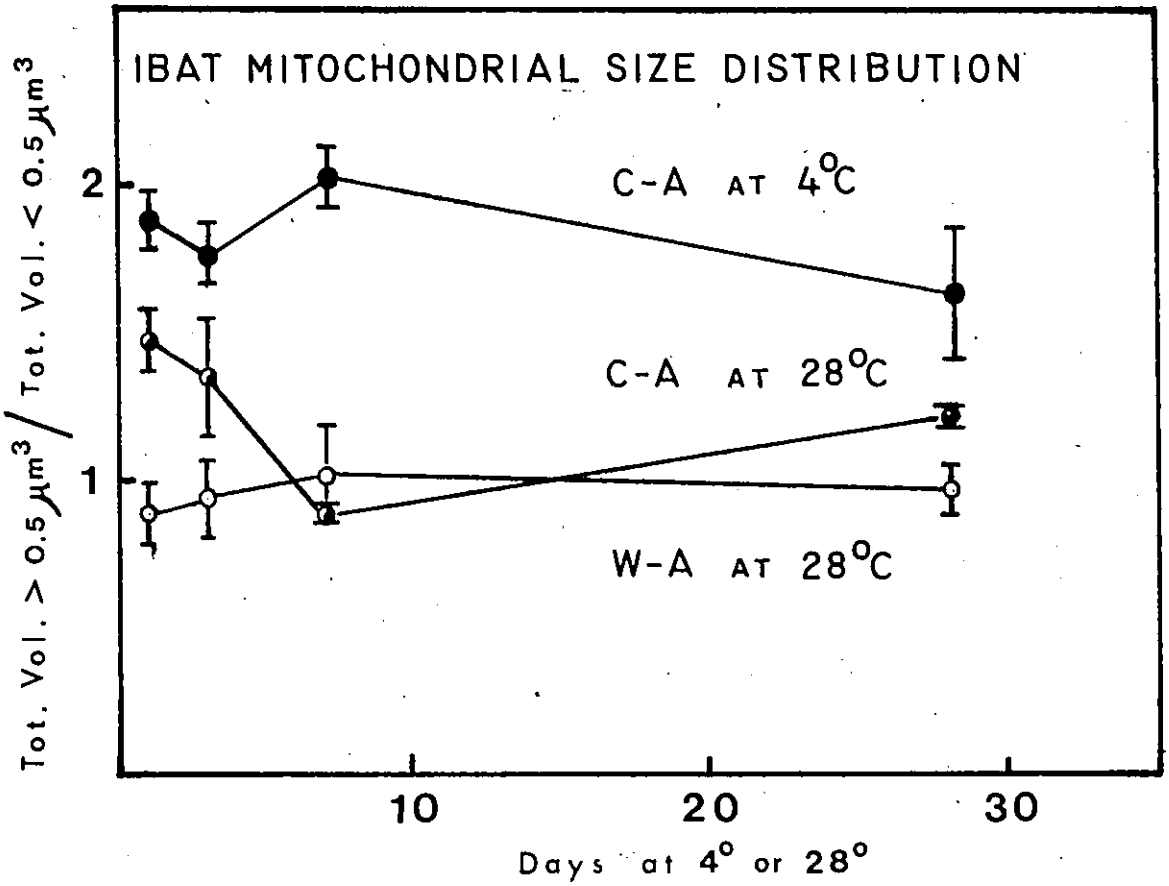


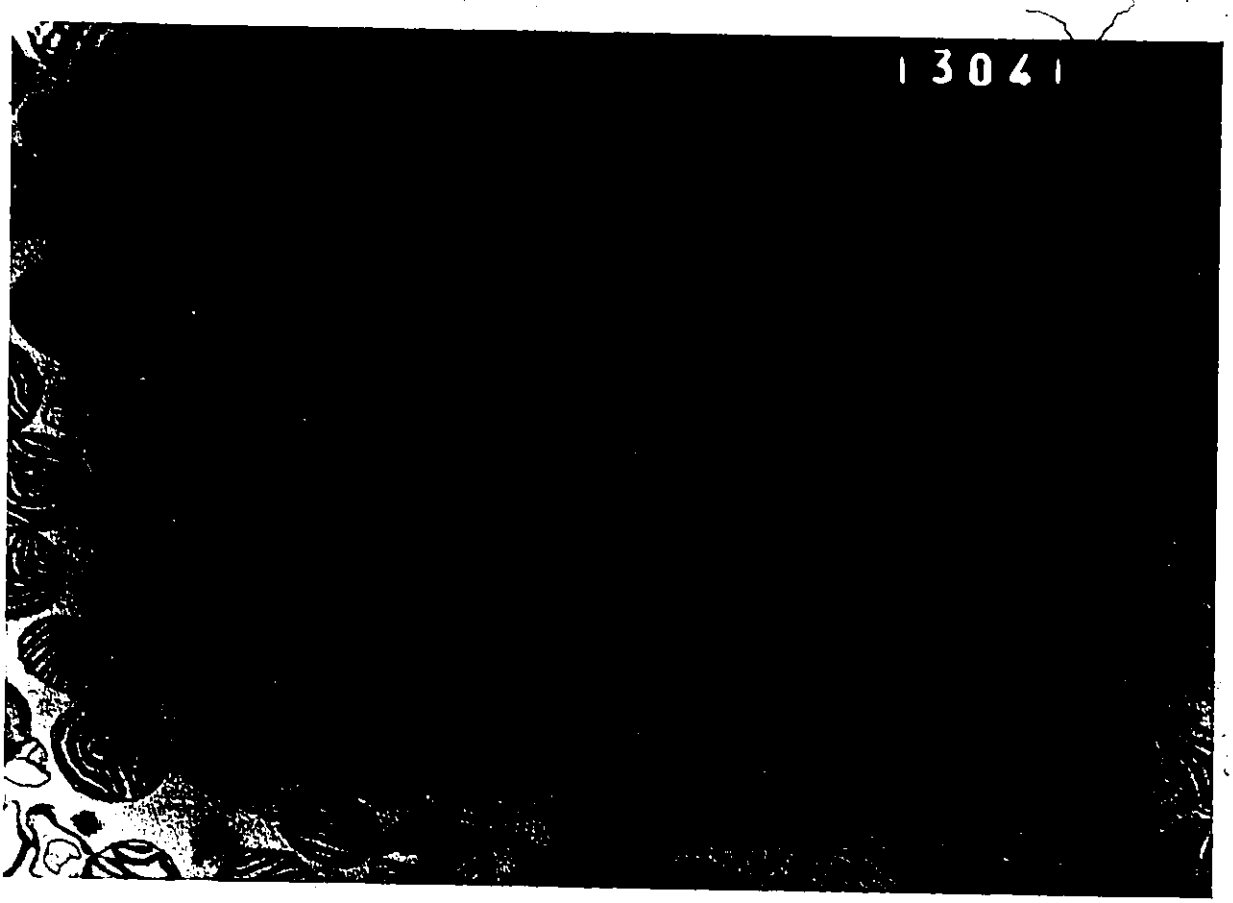
FIG. 50. Size distribution of brown adipose tissue mitochondria during readaptation to warm of rats that have been cold-acclimated for 5 weeks.

Cold-acclimated rats were either left at 4°C (C-A AT 4°C) or returned to 28°C (C-A AT 28°C) for the times indicated. For comparison, the results from corresponding warm-acclimated rats (W-A AT 28°C) were also included. Values are the means ± S.E. of 3 observations.

FIG. 51. IBAT mitochondria isolated from cold-acclimated rats (5 weeks at 4°C) that have been returned to warm (28°C) for 1 day. (x 16 000)

FIG. 52. IBAT mitochondria isolated from cold-acclimated rats (5 weeks at 4°C) that have been returned to warm (28°C) for 3 days. (x 16 000)

51



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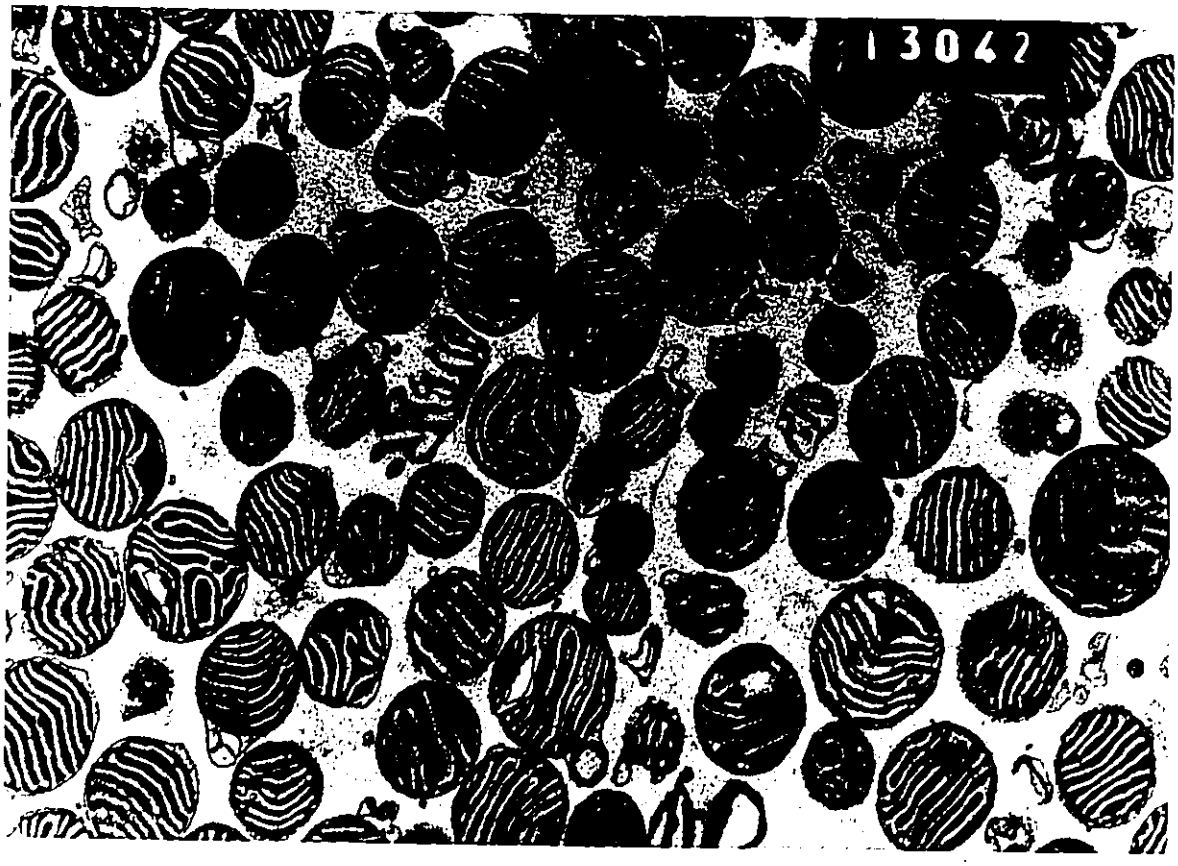

FIG. 53. IBAT mitochondria isolated from cold-acclimated rats (5 weeks at 4°C) that have been returned to warm (28°C) for 7 days. (x 16 000)

FIG. 54. IBAT mitochondria isolated from cold-acclimated rats (5 weeks at 4°C) that have been returned to warm (28°C) for 28 days. (x 16 000)

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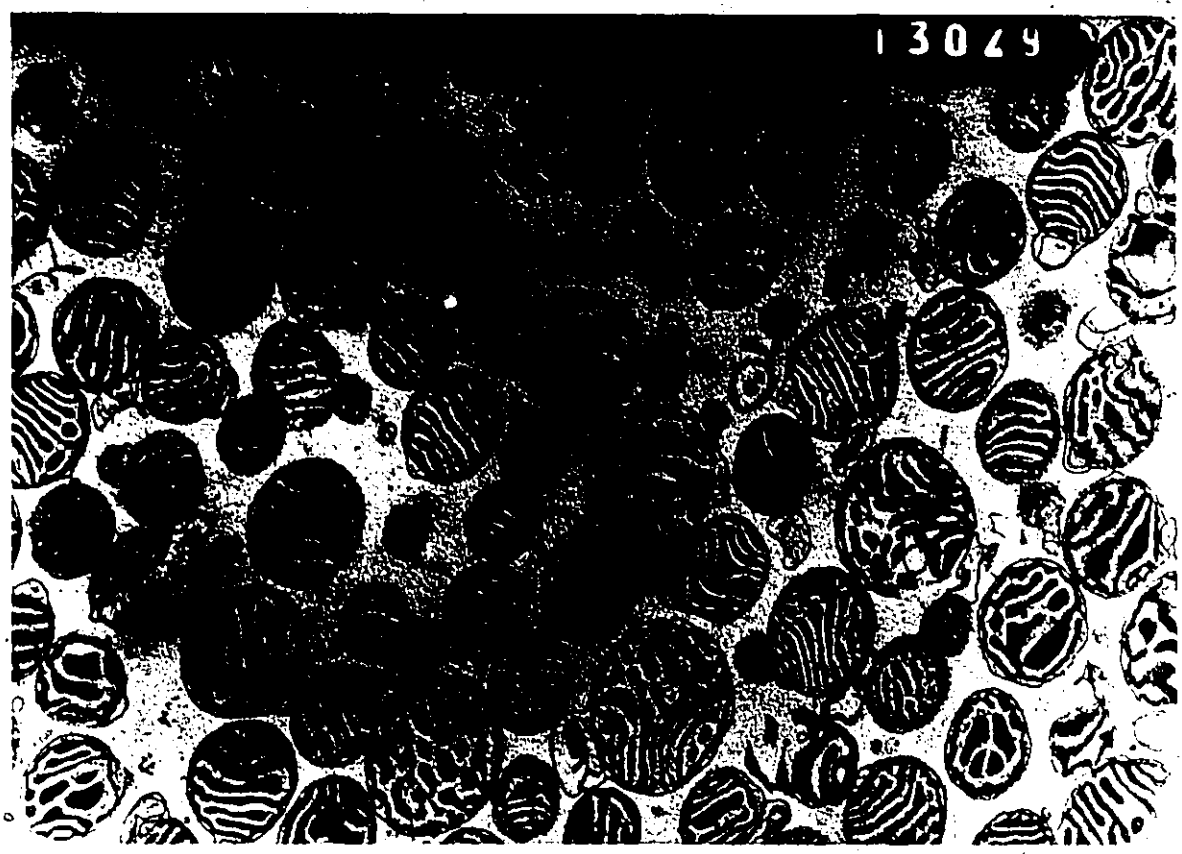

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FIG. 55. IBAT mitochondria from warm-acclimated rats (5 weeks at 28°C) that have been kept at 28°C for 28 days. (W-A control) (x 16 000)

FIG. 56. IBAT mitochondria isolated from cold-acclimated rats (5 weeks at 4°C) that have been kept at 4°C for 28 days. (C-A control) (x 16 000)

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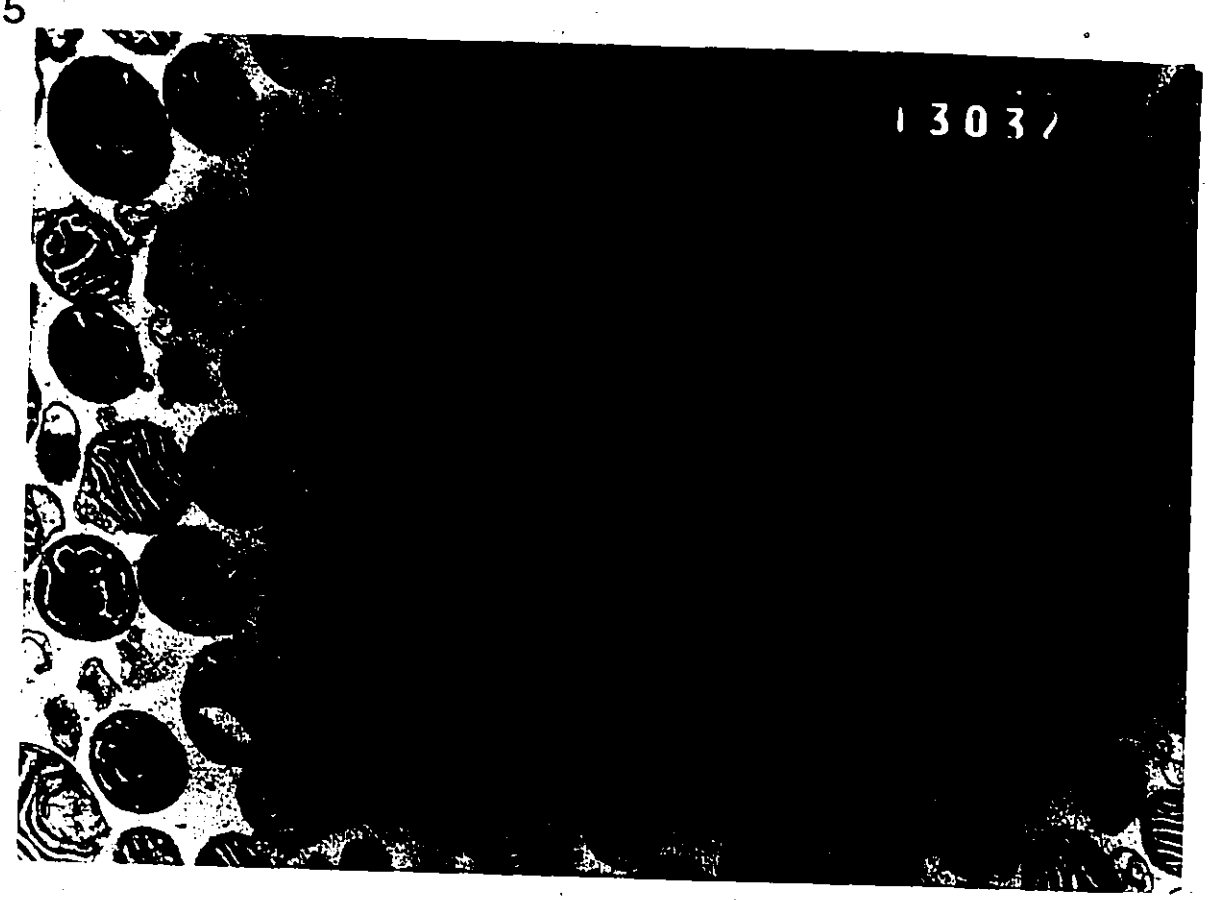

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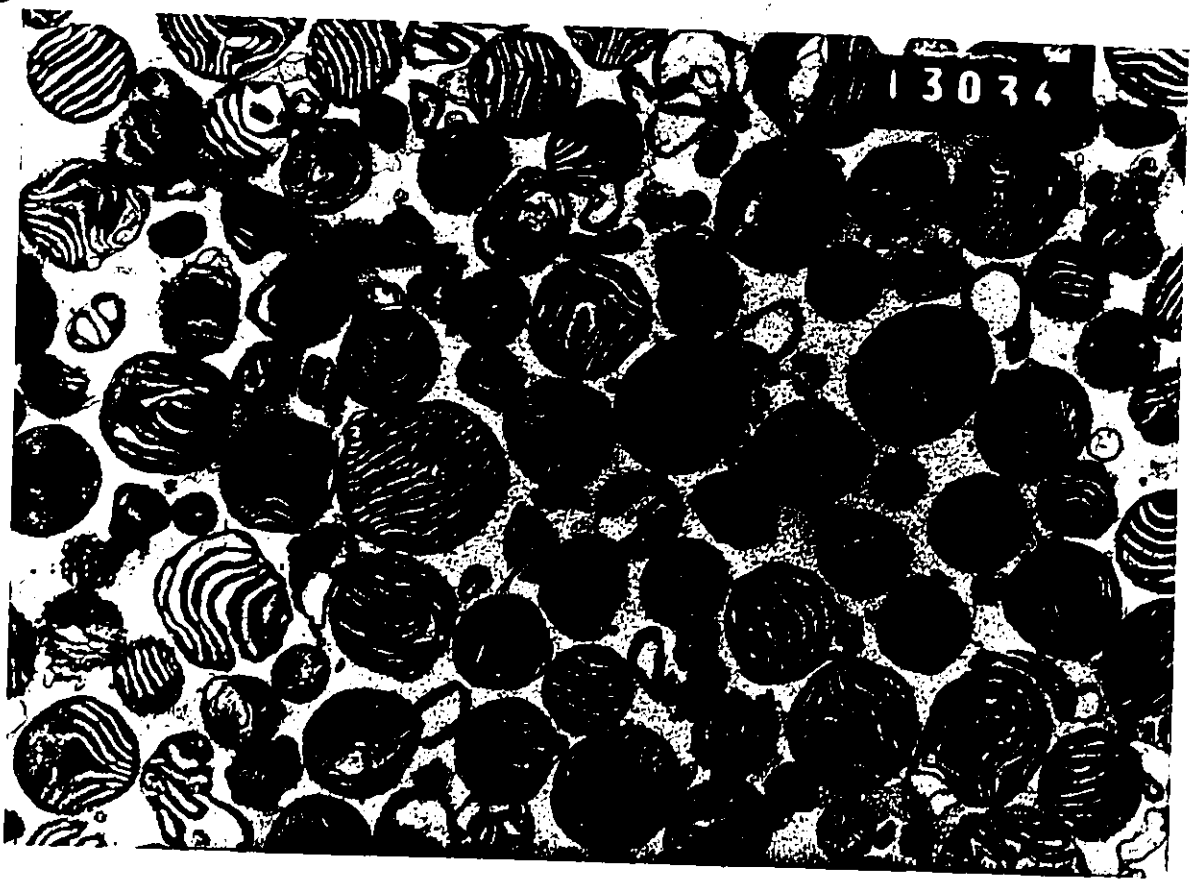

Cold-acclimated rats when placed in a warm environment (>25°C) experience heat stress (86), have a greater resting metabolic rate (83, 86, 167) and a higher heat production (61) although the plasma NE concentration is very low and not different in the cold- or warm-acclimated rats that are exposed to 28°C (58). In addition, it was postulated in a previous section of this thesis (Part I, section C) that it might not be possible to recouple BAT mitochondria of C-A rats because the amount of proton conductance pathways may be in excess of the amount of purine nucleotides available for binding in the cytoplasm. It is thus possible that rapid and general degradation of BAT is needed in order to stop the energy waste resulting from the presence of a thermogenic tissue at a time when it is not necessary anymore.

Conclusions: The change in mitochondrial size and structure seen in BAT mitochondria of C-A rats regresses in parallel with the regression of the changes in composition and purine nucleotide binding during de-acclimation of cold-acclimated rats. No biphasic change is seen, such as occurs during acclimation to cold. It appears likely that tissue degradation underlies the regression of the cold-induced changes.

PART III. THE WAY IN WHICH THE CHANGES IN BAT MITOCHONDRIA OF C-A RATS ARE BROUGHT ABOUT DURING ACCLIMATION TO COLD

Specific changes in mitochondrial structure and composition occur during adaptation of rats to cold. In this Section (III), three experimental approaches are used to try to elucidate the mechanism by which changes in BAT mitochondria are brought about during acclimation to cold:

a. To what extent are changes in protein synthesis involved in the acute and adaptive phase of cold acclimation?

b. What is the hormone mediating the mitochondrial changes in BAT of rats acutely or chronically exposed to cold?

c. Is it possible to reproduce unmasking of purine nucleotide binding sites in vitro with isolated mitochondria in order to elucidate the mechanism by which such unmasking may take place?

A. ROLE OF PROTEIN SYNTHESIS IN COLD ACCLIMATION

Upon exposure to cold, an increase in purine nucleotide binding occurs rapidly and precedes the increase in the 32 000 polypeptide known to be the binding site of the purine nucleotides (see Part II, section A-1). Mitochondrial structural changes are also observed during the acute phase of cold exposure followed by a progressive increase in inner membrane material in the adaptive phase (see Part II, section A-2).

It is well known that both cytoplasmic and mitochondrial protein

synthesis contribute to mitochondrial formation. It is possible to differentiate between the two by the use of two specific inhibitors. Cycloheximide inhibits cytoplasmic protein synthesis while oxytetracycline inhibits mitochondrial protein synthesis (215). Cycloheximide is known to block the cold-induced increase in BAT mass and protein content (130) while oxytetracycline inhibits only the mitochondrial proliferation as indicated by the lack of increase of brown fat cytochrome oxidase content in cold-exposed oxytetracycline-treated rats (123) despite an increase in total tissue protein in these animals.

Objective: The objective of this work is to investigate to what extent protein synthesis is involved during acclimation to cold, most particularly in the early phase of cold stress and to determine whether the 32 000 polypeptide is of mitochondrial or cytoplasmic origin.

Method: In order to study the role of protein synthesis, attempts were made to prevent the cold-induced changes by using the inhibitors cycloheximide and oxytetracycline. Short and long term experiments were performed in which the effects of protein synthesis inhibitors on the cold-induced changes in BAT mitochondria were studied.

In short term experiments, rats were injected intraperitoneally with a saline solution of cycloheximide (CHX) (4 mg/ml) at a dose of 1.5 mg/100 g B.W. two hours before cold exposure (256). The time spent in the cold (4°C) was one hour. Saline (SAL) injected rats served as controls. Four young (200 g) warm-acclimated rats were used per experiment. They were divided into 4 groups (1 rat / group): CHX-treated, cold-exposed; CHX-treated, warm-exposed; SAL-treated, cold-exposed; SAL-treated, warm-

exposed. In order to assess the effectiveness of the cycloheximide treatment, a single injection of tritiated leucine (25 µCi/100 g B.W. diluted with saline so that the total volume injected was 0.2 - 0.4 ml) was given to young W-A rats (200 g) two hours after the cycloheximide injection and the rats killed one hour later. The mitochondria from the BAT were isolated as usual and the protein prepared for counting of radioactivity as described previously (27).

In long term experiments, rats were injected either with cycloheximide (first dose 0.15 mg/100 g, subsequent two doses 0.75 mg/100 g B.W. in saline injected intraperitoneally) at 12 hour intervals, or with oxytetracycline (Terramycin®, 100 mg/kg injected intramuscularly) twice daily. Control rats received injections of saline by the same route and at the same time as the experimental animals received the protein synthesis inhibitors. Rats treated with cycloheximide and their controls were killed after one day of treatment at 4°C or 28°C, (the rats at 4°C did not survive for more than one day). In a first series of experiments, rats were transferred to 4°C only 2 hours after the first injection and were killed 2 hours after the third one. In a second series of experiments, rats which had already been cold - exposed for 24 hours received cycloheximide injections for their second 24 h at 4°C. Rats treated with oxytetracycline were transferred to 4°C only 12 hours after the first injection; they and their controls were killed after one week of treatment. Eight to twelve rats were used per experiment. The animals were divided into 4 groups: inhibitor-treated, warm- or cold-exposed; saline-treated, warm- or cold-exposed. Homogenization of the tissue, isolation of mitochondria and measurements of protein content, cytochrome oxidase (tissue), purine nucleotide binding and peptide pattern (mitochondria) were done as described

previously.

Results and discussion: The rapid increase in purine nucleotide binding caused by exposure of rats to cold for one hour was not prevented when the rats were pre-treated with cycloheximide (Table II, Fig. 57). The dose of cycloheximide used in this experiment (1.5 mg/100 g) was sufficient to inhibit by 80% the incorporation of radioactive leucine into BAT mitochondrial membrane proteins (Table III). Use of larger doses of cycloheximide (10 mg/100 g), sufficient to inhibit leucine incorporation by 95.2%, did not give useful results for reasons that will be explained in the next section. Cycloheximide has also an inhibitory effect on the blood flow to the tissue since the tissue oedema characteristic of short term cold exposure was absent in the cycloheximide treated rats. Although it is not possible to inhibit protein synthesis completely by the use of cycloheximide, it seems unlikely that protein synthesis in the cytosol is required for the rapid cold-induced increase in purine nucleotide binding.

In order to determine whether protein synthesis is necessary for the slow increase in the 32 000 M.W. polypeptide which occurs during acclimation to cold, longer term experiments were needed. Cycloheximide also fails to block the increase in purine nucleotide binding in BAT mitochondria of rats exposed to cold for 24 hours (Table IV, Fig. 58). There is no marked effects of either cold or cycloheximide on the BAT mainly because of the short time of treatment with cycloheximide and the incompleteness of the inhibition (Table III) at the doses used in these experiments. However, the fact that the cycloheximide-treated and cold-exposed rats lost weight (Table IV) and died if treated for more than 24

hours suggests that they were markedly impaired by the treatment. Nevertheless, cycloheximide treatment had an effect on the second day of cold exposure (Table V, Fig. 58). After the initial CHX-insensitive increase in purine nucleotide binding had occurred, the CHX treatment did prevent any further increase in purine nucleotide binding during the second day of exposure to cold. Unfortunately, there was insufficient cold-induced changes in the 32 000 M.W. polypeptide in these experiments for any conclusions to be drawn about a possible inhibitory effect of CHX in the cold-induced increase in the proportion of the polypeptide. It was however not possible to perform longer term experiment with these inhibitors due to the high toxicity of these compounds. The prevention of any further increase in purine nucleotide binding during the second day of cold exposure by CHX suggests that cytosolic protein synthesis is required at this time and is probably associated with the start of the increase in the 32 000 M.W. polypeptide. Dittmar E. and Himms-Hagen J. (unpublished data) have demonstrated a preferential incorporation of amino acids into polypeptides in the region of 32 000 during this phase of acclimation to cold.

Mitochondrial protein synthesis is unlikely to be involved in the increase in the concentration of the proton conductance pathway in mitochondria of C-A rats. The effects of oxytetracycline on BAT of seven day cold-exposed rats are presented in Table VI (Fig. 59). The oxytetracycline treatment prevented the increase in cytochrome oxidase and reduced the increase in total protein. It did not however, prevent the increase in purine nucleotide binding and in the 32 000 M.W. polypeptide. Thus, the slow adaptive increase in the concentration of proton conductance pathway during acclimation to cold appears to require cytosolic but not mitochondrial protein synthesis. Both are however necessary for the tissue hypertrophy

TABLE II. Effect of cycloheximide (1.5 mg/100 g, 2 h before) on the increase in purine nucleotide binding induced by cold exposure for 1 h

	No. of experiments	Saline		Cycloheximide	
		Warm	Cold	Warm	Cold
Body weights (g)	9	220.3 ± 12.3	217.2 ± 9.4	213.2 ± 10.3	218.3 ± 11.5
IBAT weight (g)	9	0.220 ± 0.017	0.307 ± 0.017*	0.235 ± 0.013	0.235 ± 0.011†
IBAT mitochondria					
[^{3}H] GDP binding (nmol/mg protein)	9	0.063 ± 0.012	0.128 ± 0.008*	0.044 ± 0.006	0.097 ± 0.014*
[^{3}H] ADP binding (nmol/mg protein)	9	0.080 ± 0.020	0.128 ± 0.024*	0.033 ± 0.008	0.113 ± 0.016*

Note: Values are means ± SE.

* The value is significantly different ($P < 0.05$ or less) from the value for similarly treated rats in the warm.

† The value is significantly different ($P < 0.05$ or less) from the value for saline-injected rats at the same temperature.

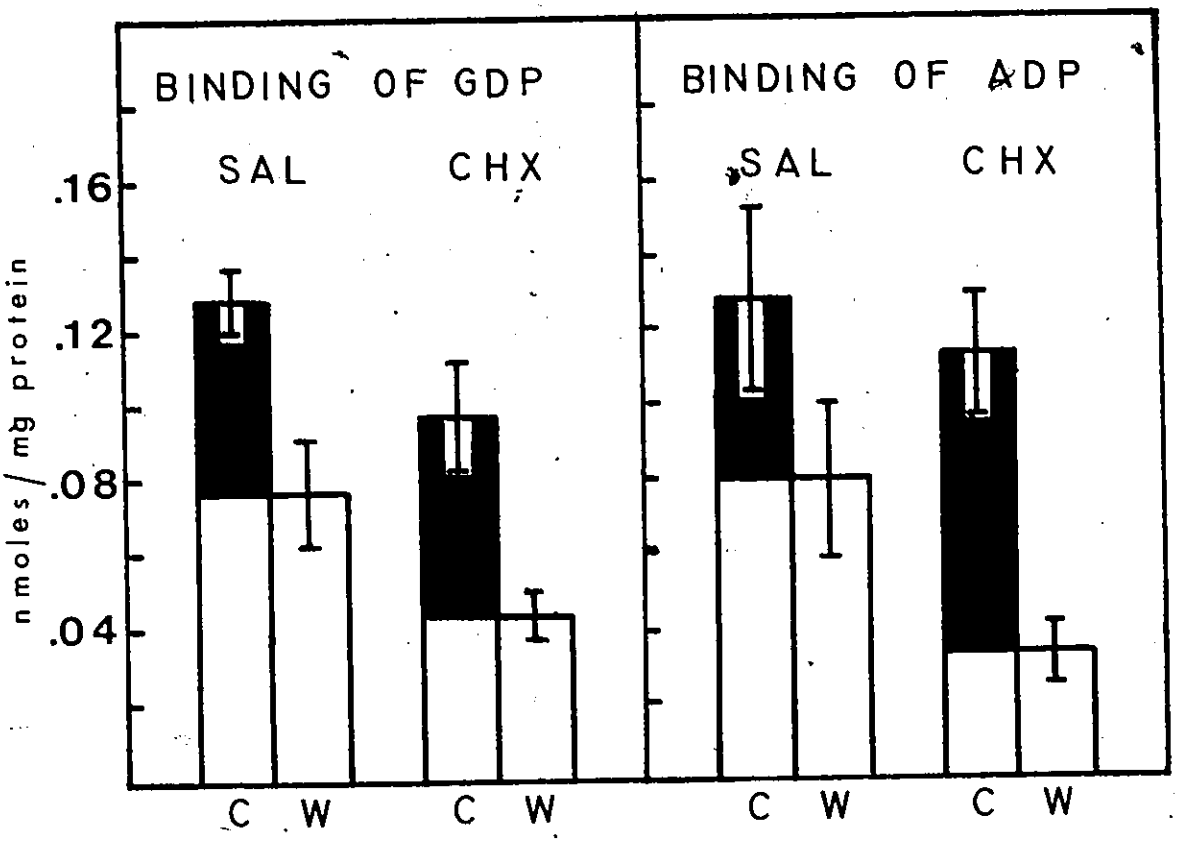

FIG. 57. Effect of cycloheximide (1.5 mg/100 g B.W., 2 hours before) on the increase in purine nucleotide binding induced by cold-exposure for 1 hour.

Saline (SAL) or cycloheximide (CHX) treated rats were exposed to 4°C (C) or 28°C (W) for one hour. The values are the means ± S.E. of 9 separate experiments.

TABLE III. Inhibition of [^{3}H]leucine incorporation into IBAT mitochondrial proteins by cycloheximide

Experiment	Cycloheximide	No of experiments	% Inhibition
1	1.5 mg/100 g, 2 h before	3	80.8 ± 8.5
2	10 mg/100 g, 2 h before	3	95.2 ± 0.7
3	0.15 mg/100 g, 2 h before	2	80.4 ± 10.5
	0.15 mg/100 g, 22 h before and 0.075 mg/100 g, 10 h before	3	19.9 ± 10.2

Note: Values are means ± SE.

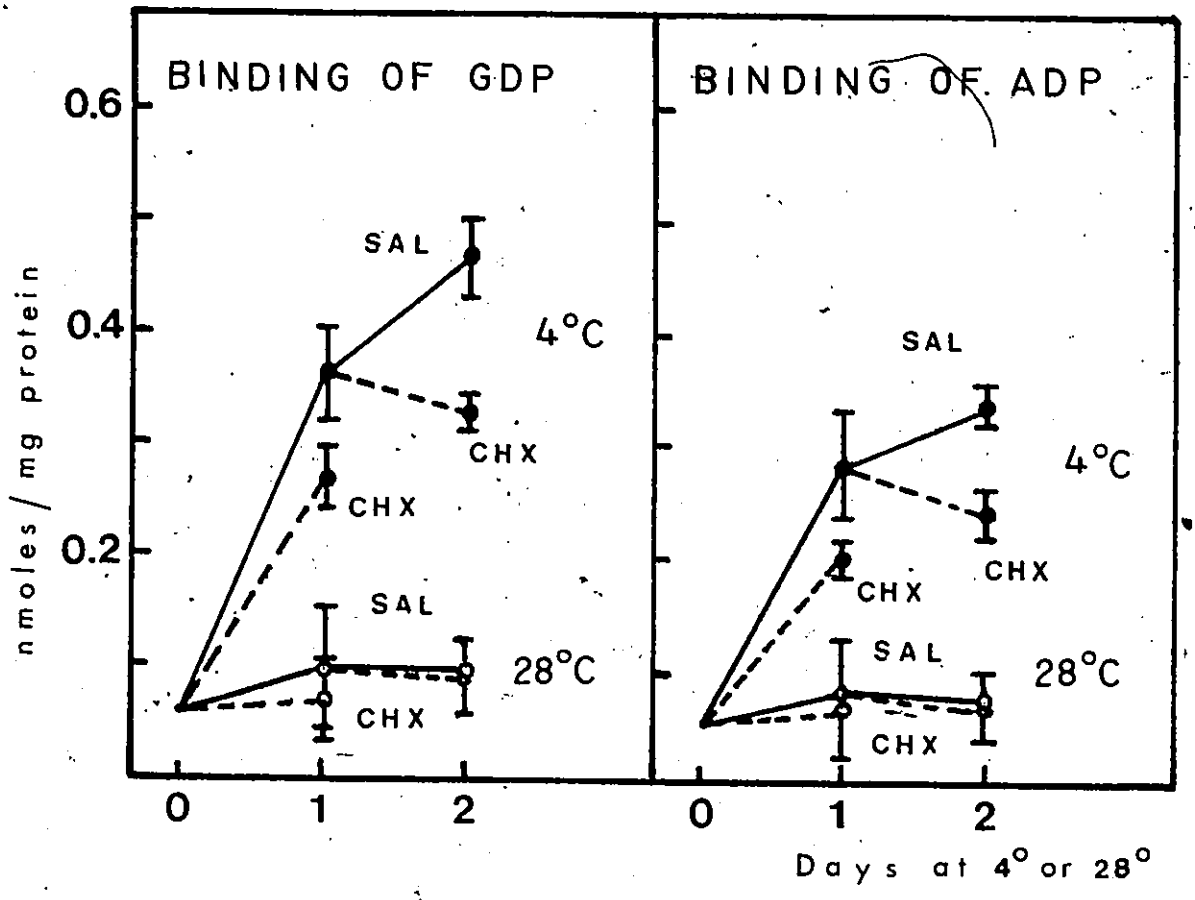

FIG. 58. Effect of cycloheximide on the cold-induced increase in GDP or ADP binding by IBAT mitochondria.

Rats were treated with cycloheximide or saline either starting before they were exposed to cold (4°C) and continuing during the first 24 hours in the cold (CHX 0-1 day, Saline 0-1 day) or starting after they had been exposed to cold for 1 day and continuing during the second day in the cold (CHX 1-2 days, Saline 1-2 days).

Cold-exposed rats are denoted by solid circles and warm-acclimated (28°C) rats by open circles. The values are the means ± S.E. of 3 separate observations.

TABLE IV. Effect of cycloheximide treatment on the first day of cold exposure on cold-induced changes in brown adipose tissue

	Saline		Cycloheximide	
	Warm	Cold	Warm	Cold
Body weights (g)				
Start	203.0 ± 4.8	198.1 ± 7.5	194.4 ± 6.1	200.2 ± 4.2
Finish	204.9 ± 8.4	195.2 ± 11.4	184.1 ± 10.4	177.6 ± 6.7
IBAT				
Wet weight (g)	0.250 ± 0.019	0.244 ± 0.026	0.215 ± 0.009	0.188 ± 0.014
Total protein (mg)	17.43 ± 0.93	20.08 ± 0.85	16.95 ± 1.35	17.91 ± 1.23
Total cytochrome oxidase (μg atoms O/min)	61.19 ± 7.38	55.91 ± 12.82	62.28 ± 1.01	55.13 ± 13.36
IBAT mitochondria				
[^{3}H] GDP binding (nmol/mg protein)	0.102 ± 0.049	0.371 ± 0.044*	0.075 ± 0.016	0.267 ± 0.022*
[^{3}H] ADP binding (nmol/mg protein)	0.080 ± 0.049	0.280 ± 0.044*	0.06 ± 0.029	0.200 ± 0.005*
Proportion of total polypeptide in 30 000 - 35 000 region	11.31 ± 0.31	11.84 ± 0.40	11.43 ± 0.14	12.74 ± 0.45

Note: Body weights are those before the first injection (Start) and after 24 h at 28°C (Warm) or 4°C (Cold) (Finish). Values are the means of three experiments. Values are means ± SE.

* Values shown are significantly different ($P < 0.05$ or less) from values for similarly treated rats in the warm.

TABLE V. Effect of cycloheximide treatment on the 2nd day of cold exposure on cold-induced changes in brown adipose tissue

	Saline		Cycloheximide	
	Warm	Cold	Warm	Cold
Body weights (g)				
Start	194.6± 6.0	190.9 ± 8.6	189.2 ± 4.9	190.8 ± 10.1
Finish	209.9± 6.8	184.9 ± 5.1*	187.6 ± 4.7	172.1 ± 10.2
IBAT				
Wet weight (g)	0.283± 0.017	0.214 ± 0.021	0.234 ± 0.025	0.188 ± 0.017
Total protein (mg)	13.9± 0.6	19.5 ± 3.0	12.6 ± 0.8	15.5 ± 1.3
Total cytochrome oxidase (µg atoms O/min)	79.7± 4.1	51.7 ± 1.4*	68.9 ± 9.1	53.1 ± 2.2
IBAT mitochondria				
[^{3}H] GDP binding (nmol/mg protein)	0.098± 0.030	0.473 ± 0.028*	0.095 ± 0.013	0.328 ± 0.011*†
[^{3}H] ADP binding (nmol/mg protein)	0.073± 0.019	0.336 ± 0.017*	0.058 ± 0.010	0.237 ± 0.013*†
Proportion of total polypeptides in 30 000 - 35 000 region	13.90 ± 0.57	15.45 ± 0.91	14.63 ± 0.67	16.06 ± 0.66

Note: Values are the means of three experiments ± SE.

* These values are significantly different ($P < 0.05$ or less) from those for similarly treated rats in the warm.

† These values are significantly different ($P < 0.05$ or less) from those for saline-treated rats at the same temperature.

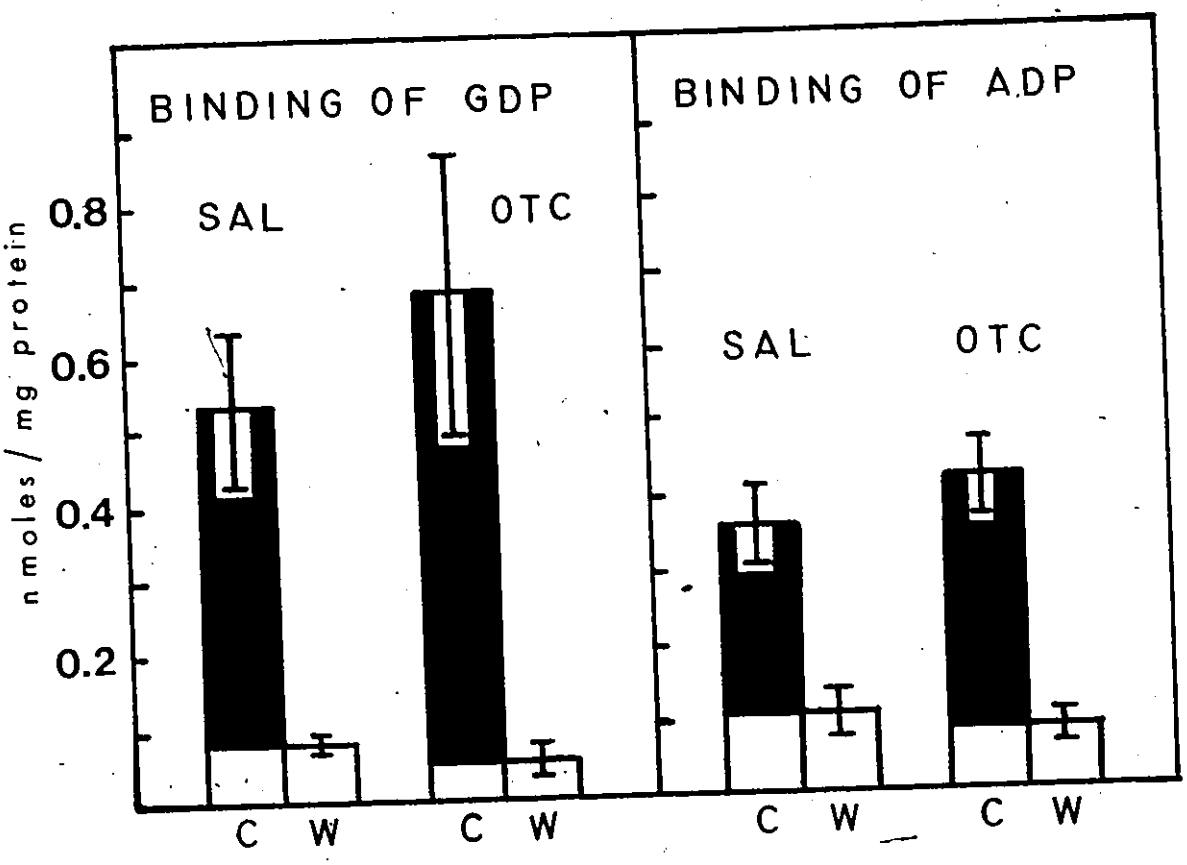

FIG. 59 Effect of oxytetracycline on cold-induced increase in purine nucleotide binding to IBAT mitochondria.

Saline (SAL) or oxytetracycline (OTC) treated rats exposed to 4°C (C) or 28°C (W) for 7 days. The values are the means ± S.E. of 3 separate experiments.

TABLE VI. Effect of oxytetracycline on cold-induced changes in brown adipose tissue

	Saline		Oxytetracycline	
	Warm	Cold	Warm	Cold
Body weights (g)				
Start	185.3 ± 5.7	184.7 ± 7.4	193.6 ± 2.3	180.4 ± 2.0
Finish	239.4 ± 7.8	202.0 ± 8.8*	181.2 ± 2.4†	185.6 ± 6.6
IBAT				
Wet weight (g)	0.213 ± 0.007	0.483 ± 0.016*	0.161 ± 0.031	0.348 ± 0.009*†
Total protein (mg)	15.76 ± 1.13	46.11 ± 3.97*	12.03 ± 2.4	30.94 ± 2.05*†
Total cytochrome oxidase (µg atoms O/min)	60.49 ± 2.14	91.19 ± 13.20	21.99 ± 1.37†	29.07 ± 4.26†
IBAT mitochondria				
[^{3}H] GDP binding (nmol/mg protein)	0.078 ± 0.006	0.525 ± 0.095*	0.053 ± 0.008	0.677 ± 0.177*
[^{3}H] ADP binding (nmol/mg protein)	0.105 ± 0.034	0.361 ± 0.049*	0.076 ± 0.020	0.417 ± 0.049*
Proportion of total polypeptides in 30 000 - 35 000 region	12.65 ± 0.21	14.11 ± 0.41*	13.75 ± 0.26	15.11 ± 0.51*

Note: Values are means ± SE.

* Values shown are significantly different ($P < 0.05$ or less) from those for similarly treated rats in the warm.

† Values shown are significantly different ($P < 0.05$ or less) from those for rats treated with saline at the same temperature.

and the large proliferation of mitochondria during acclimation to cold.

Conclusions: These results support the hypothesis of an unmasking of proton conductance sites occurring during the first hours of exposure to cold. Protein synthesis is not required for the rapid response of BAT mitochondria to acute cold exposure. The adaptive phase of acclimation to cold obviously requires protein synthesis for both the tissue hypertrophy and the mitochondrial proliferation. The increase in the concentration of proton conductance pathway (formation of new sites) is dependent on cytosolic protein synthesis.

B. NATURE OF HORMONAL MEDIATOR OF COLD ACCLIMATION

Cold acclimation is a biphasic process. One or several hormones may be responsible for the various changes in BAT mitochondrial structure and composition during either the acute or adaptive phase of cold acclimation. This section is concerned with the nature of the mediators of these changes. For reasons that will be outlined below, norepinephrine appears to be the most likely candidate for mediating the changes in both the acute and the adaptive phases. This section is divided in two parts which describe the effects on BAT mitochondrial composition and structure of short and long term administration of norepinephrine.

1. Acute effect of norepinephrine

It is known that large amounts of norepinephrine are secreted within

the BAT from sympathetic nerve endings in response to cold (58, 60). Isolated brown adipocytes from rat or hamster respond to NE by an increase in respiratory rate (23, 192). This increase in respiratory rate is completely abolished when the administration of NE is followed by propanolol, a β-blocking agent (23, 192). Similarly, intravenous infusion of catecholamines results in an increase in the metabolic rate of the rat which returns to basal value very rapidly when the infusion is stopped (124) (see Fig. 1).

Upon acute cold exposure, there is an unmasking of proton conductance pathways characterized by an increase in purine nucleotide binding (see Part II, A-1), independent of protein synthesis (Part III, A) and possibly related to changes in mitochondrial structure (Part II, A-2) which is believed to contribute to increase BAT thermogenesis.

Objective: The purpose of this work is to find out whether NE might mediate the effect of cold stress to unmask purine nucleotide binding sites.

Method: For study of the acute effect of NE, it was necessary to infuse NE intravenously into unanaesthetized, conscious rats with an indwelling venous cannula. The weight of the rats before the surgical implantation of the cannula was 172 ± 2 g. They were anaesthetized with Innovar-Vet (0.03 ml/100 g B.W. intramuscularly) for surgery. Their jugular vein was cannulated (20), a solution of 500 units heparin per 10 ml saline being used throughout the procedure. At the end of surgery, 0.1 ml of penicillin (Penlong S, containing 10 000 units Benzathine penicillin G and 10 000 units Procaine penicillin G) was given intra-

muscularly. After 4-10 days, rats showing normal weight gain and no sign of leakage from their caps were transferred to the test chamber (275). The weight of the rats at the time of the experiments was 214 ± 5 g. They were transferred to the test chamber 1 to 2 hours before the start of the infusion; then, 0.25 µg NE (base) / 100 cm^2-minute was infused for 1 hour. The surface area of the rat was obtained from the relation: Area (cm^2) = K x body weight $^{2/3}$, (where K = 7.5, Biology Data Book, FASEB 1964, p. 121). The rate of infusion was 0.0206 ml/min. A similarly operated rat infused with saline was used as a control.

In experiments where the effects of protein synthesis inhibitors were studied, rats were injected intraperitoneally with a saline solution of cycloheximide (CHX)(4 mg/ml) at a dose of 1.5 mg/100 g B.W. two hours before the start of the infusion. Purine nucleotide binding and size distribution measurements as well as electron microscopy were done on isolated mitochondria from IBAT as described previously.

Results and discussion: The infusion of NE at a dosage sufficient to induce a maximum increase in metabolic rate (131), results in a large increase in purine nucleotide binding by BAT mitochondria (Fig. 60). The change is completely reversible and purine nucleotide binding is not different from that of saline infused rats by 1 hour after the end of the infusion. It should be noted that this effect of NE is demonstrable only when conscious rats with indwelling cannulas are used. In preliminary experiments, rats were infused with NE or saline via a cannula in the tail vein while they were anaesthetized with pentobarbital or while they were conscious shortly after a cannula had been placed in the tail vein under ether anaesthesia. In these experiments, the purine nucleotide

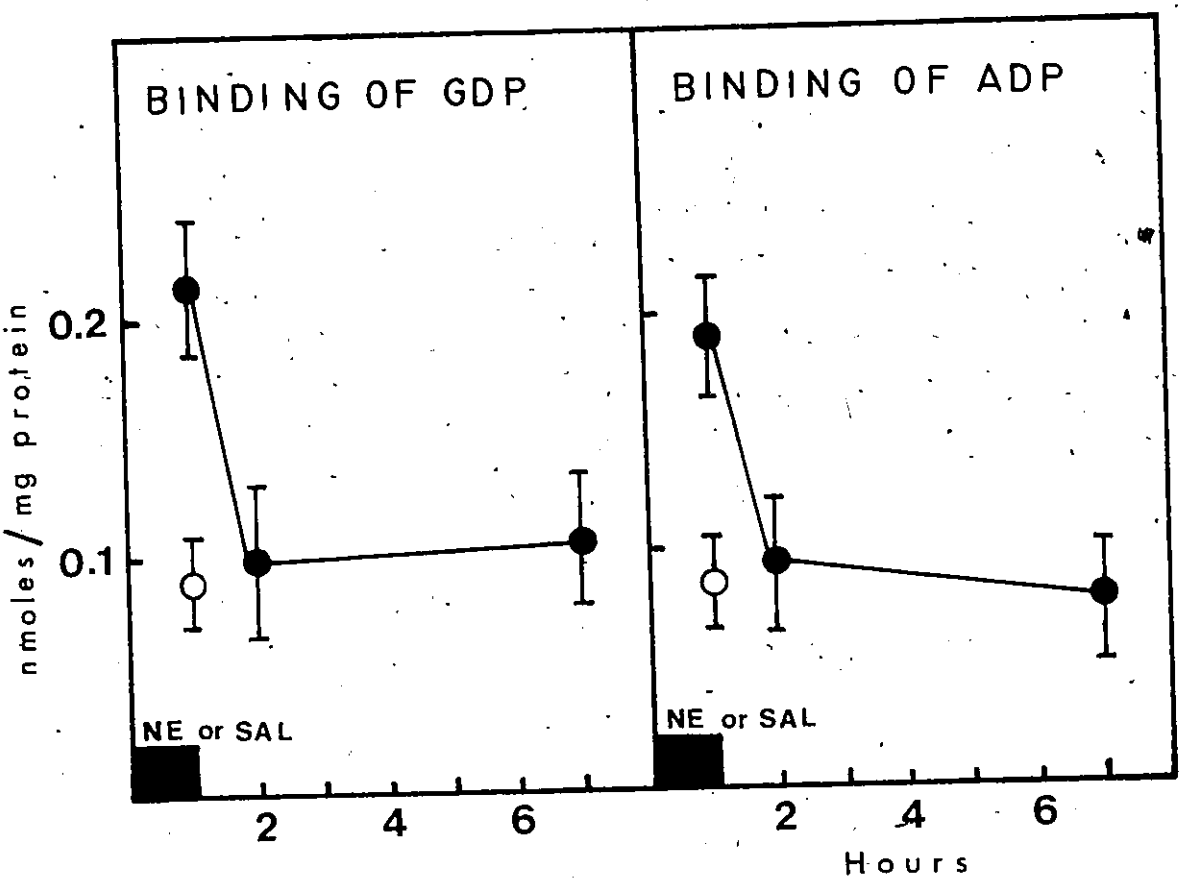

FIG. 60 Effect of intravenous infusion of NE on purine nucleotide binding by BAT mitochondria.

The animals were infused with saline (SAL; open circle) or norepinephrine (NE; solid circles) for 1 hour (black bar). The values are the means ± S.E. of 3-5 observations.

The actual GDP binding data are:
SAL at 1 h, 0.087 ± 0.018 nmoles/mg protein
NE at 1 h, 0.213 ± 0.029, $p < .025$
NE at 2 h, 0.096 ± 0.03 , NS
NE at 7 h, 0.104 ± 0.029, NS

The actual ADP binding date are:
SAL at 1 h, 0.083 ± 0.018 nmoles/mg protein
NE at 1 h, 0.188 ± 0.026, $p < 0.05$
NE at 2 h, 0.094 ± 0.027, NS
NE at 7 h, 0.079 ± 0.023, NS

Comparisons for statistical significance were taken relative to the SAL-infused rats (NS = non significant).

binding was already very high in the saline-infused rats and no additional increase occurred in response to infusion of NE. Presumably, the activation of the sympathetic nervous system, known to be caused by such experimental procedures (22, 59, 172), was sufficient to exert a maximal effect on the BAT to cause an increase in purine nucleotide binding.

Protein synthesis is not required for the increase in purine nucleotide binding upon NE infusion (Fig. 61). In rats pre-treated with cycloheximide (1.5 mg / 100 g B.W.), the infusion of NE still caused the increase in purine nucleotide binding. The dose of CHX used is sufficient to inhibit by 80% the incorporation of radioactive leucine into BAT mitochondrial proteins (Table III). The use of a larger dose of CHX (10 mg / 100 g B.W.) sufficient to inhibit leucine incorporation by 95.2% resulted in so much stress for the animal that the purine nucleotide binding was increased to a maximal level in the saline-infused rat (0.335 and 0.192 nmoles per mg protein for ^{3}H-GDP and ^{3}H-ADP binding respectively) and no further increase was seen due to the infusion of NE.

The electron micrographs of IBAT mitochondria from NE or saline-infused rats are shown in Fig. 62 and 63. The experiment has only been performed once. The appearance of mitochondria is the same for both SAL or NE-infused rats. They are however different from mitochondria of W-A rats (Fig. 32) and resemble those of 1 hour cold-exposed rats (Fig. 33). A likely explanation is that an undue stress to the SAL-infused rat resulted in a sympathetic stimulation of its BAT. There is also a small increase in mitochondrial size upon NE infusion but this did not reach statistical significance. The ratios of the total volume contributed by mitochondria of size greater than 0.5 μm^3 to the total volume contributed

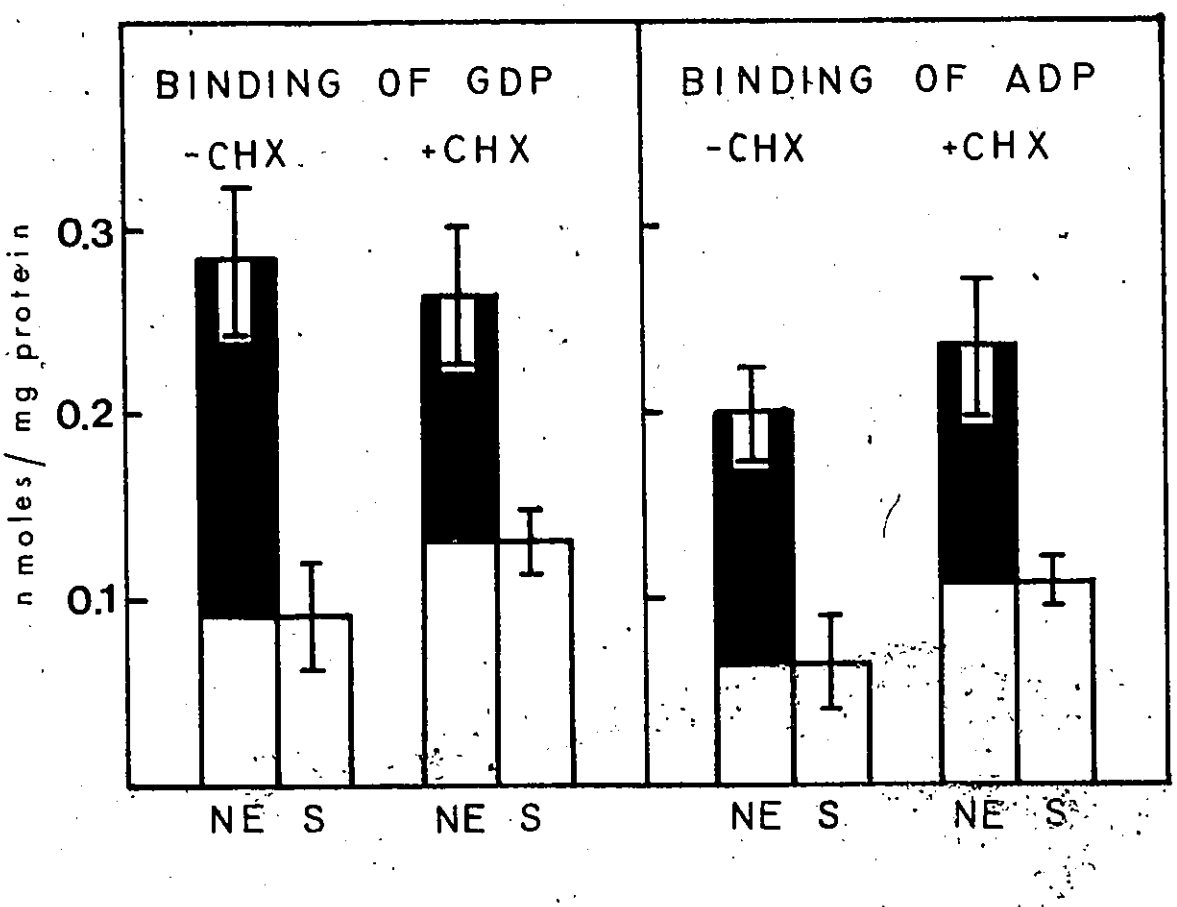

FIG. 61 Effect of cycloheximide (CHX) on the increase in IBAT mitochondrial purine nucleotide binding induced by NE infusion.

Rats pretreated with cycloheximide (CHX) or not were infused with saline (S) or norepinephrine (NE) for 1 hour.

The actual binding date are:(nmoles/mg protein)

GDP binding	- CHX		+ CHX	
NE-infused	(3)	0.284 ± 0.040	(8)	0.263 ± 0.038
SAL-infused	(3)	0.090 ± 0.028	(8)	0.133 ± 0.017
p <		0.02		0.01

ADP binding	- CHX		+ CHX	
NE - infused	(3)	0.199 ± 0.025	(8)	0.236 ± 0.037
SAL-infused	(3)	0.062 ± 0.023	(8)	0.107 ± 0.011
p <		0.02		0.005

FIG. 62. Mitochondria isolated from IBAT of a rat infused with saline for 1 hour. (x 16 000)

FIG. 63. Mitochondria isolated from IBAT of a rat infused with NE for 1 hour. (x 16 000)

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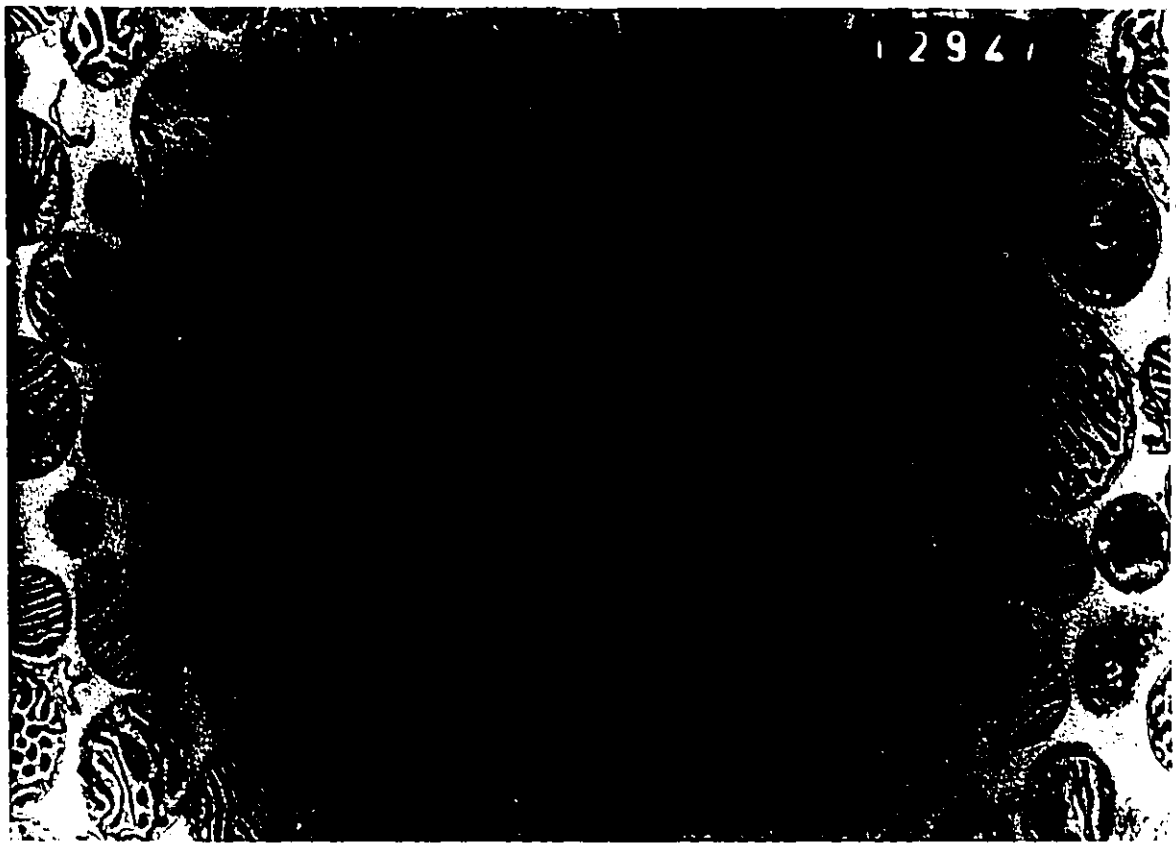

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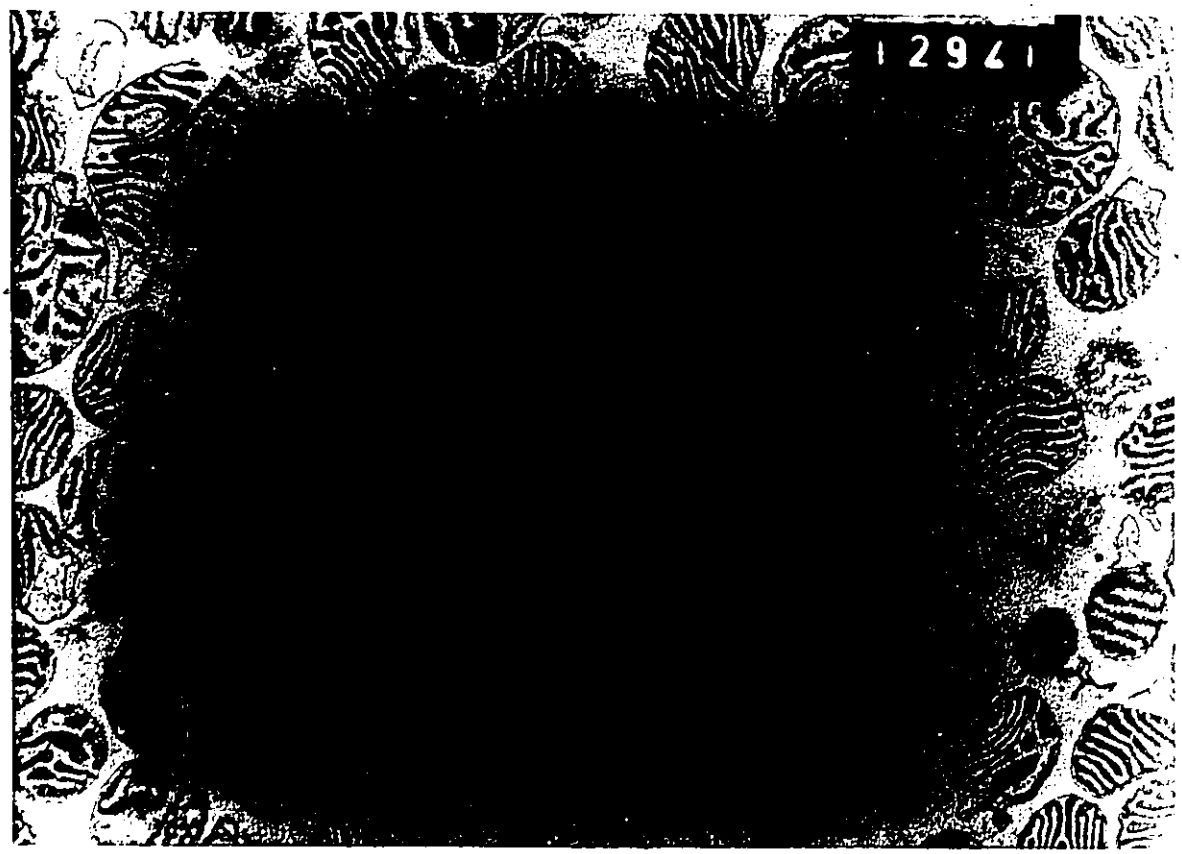


by mitochondria of size smaller than 0.5 μm^3 are 0.82 ± 0.13 for the SAL-infused rat and 1.15 ± 0.19 for the NE-infused rats (n=2, NS). Changes in mitochondrial structure have been reported on response to cold (270, 271, 279) and to NE (270, 288). A reversible swelling of the mitochondria occurs upon NE administration in newborn rats (288). Rapid changes in brown fat mitochondrial morphology of hamsters are also observed during arousal from hibernation, a time of intense sympathetic activity (102).

The physiological significance of the initial unmasking is indicated by the fact that when it fails to occur, there is also a failure to survive during exposure to cold. The genetically obese mouse (ob/ob) is known to be extremely sensitive to cold and to die in about 3 hours at 4°C due to a failure of nonshivering thermogenesis (204, 283), associated with a diminished capacity to respond to catecholamines by an increase in heat production (283). The initial response to acute cold stress is defective in obese mice in that the purine nucleotide binding to BAT mitochondria fails to increase (133). However, if the mouse is progressively acclimated to mild cold (14°C), by-passing the acute phase of cold exposure, the tissue hypertrophy and the increase in concentration of purine nucleotide binding sites occur. The mouse can now survive at 4°C for longer periods of time (Hogan S. and Himms-Hagen J., unpublished data).

Conclusions: The rapid unmasking of proton conductance pathways which occurs during acute cold stress is most probably mediated by NE. However, the possibility of an indirect action of NE through its effect on blood flow (85) can not be eliminated from these experiments. Attempts to demonstrate a direct action of NE on brown adipose tissue mitochondria in isolated adipocytes or brown fat fragments failed because of the

unsuitability of the in vitro system and other technical reasons.

The exact mechanism by which NE induces the unmasking of purine nucleotide binding sites is unknown, but it probably involves a reorganization of mitochondrial inner membranes.

2. Long-term effect of norepinephrine administration

It is known that the secretion of NE is increased and remains elevated during cold acclimation (46, 124). It is also known that chronic administration of NE to young warm-acclimated rats induces an increase in BAT mass, a proliferation of BAT mitochondria, an increased calorigenic response of the rats to catecholamines and a higher resistance to cold stress, although the changes obtained are smaller than correspondingly cold-induced changes (116, 176). The best results are obtained when NE is given in conjunction with thyroxine although the effect of thyroxine is most certainly indirect (116, 238).

In this thesis, it was confirmed that the adaptive phase of cold acclimation is characterized by an increase in BAT mass, a mitochondrial proliferation, an increase in the amount of mitochondrial inner membrane and an increase in the concentration of mitochondrial proton conductance pathways which is dependent on cytosolic protein synthesis.

Objective: The purpose of this work is to find out whether NE is responsible for the increase in the concentration of mitochondrial proton conductance pathways known to occur in the adaptive phase of cold acclimation.

Methods: For study of the effects of long term injections of hormones, rats kept at 28°C and weighing 200-250 g were injected subcutaneously twice daily for 14 or 21 days (see Tables) with a peanut oil suspension of L-arterenol (bitartrate salt) (7.5 mg / 25 ml peanut oil), thyroxine (0.47 mg/25 ml peanut oil) or a combination of both.

The doses injected were 300 µg NE (base) / kg.day and 50 µg T_4/kg.day. Rats received these daily amounts in two injections given at 8 00-10 00 hours and 18 00-20 00 hours. Rats received 0.27 - 0.4 ml of suspension. Similarly treated rats receiving only peanut oil served as controls. For comparison, rats were also left at 28°C without any injections or placed in the cold (4°C) for the same time period.

Homogenization of the tissue, isolation of mitochondria and measurements of tissue protein and cytochrome oxidase content as well as mitochondrial purine nucleotide binding and peptide pattern were performed as described previously.

Results and discussion: Treatment of rats at 28°C for 2 weeks with NE in oil resulted in the predicted increase in size, total protein and total cytochrome oxidase content of the IBAT (Table VII). There was however, no increase in the specific activity of cytochrome oxidase (relative to total tissue protein), as occurs in rats exposed to cold for 2 weeks (130). No change could be detected in the proportion of the 32 000 M.W. polypeptide and a small and variable increase in purine nucleotide binding did not reach statistical significance. These small increases were most probably due to a residual effect of a low concentration of NE left in the rats from the last injection.

When NE is given in conjunction with thyroxine, there is a greater

TABLE VII. Effect of long-term treatment (2 weeks) with norepinephrine in oil on brown adipose tissue

	No. of experiments	Control	Noradrenaline	P
Body weights (g)				
Start	9	207.8 ± 9.9	210.6 ± 11.8	NS*
Finish	9	303.5 ± 8.4	298.3 ± 9.9	NS
IBAT				
Wet weight (g)	9	0.280 ± 0.014	0.428 ± 0.025	< 0.001
Total protein (mg)	9	22.0 ± 0.90	36.6 ± 3.5	< 0.001
Total cytochrome oxidase (µg atoms O/min)	6	183.6 ± 20.4	307.1 ± 33.8	< 0.002
Specific activity of cytochrome oxidase (µg atoms O/min-mg protein)	6	8.61 ± 0.68	9.63 ± 1.26	NS
IBAT mitochondria				
[^{3}H] GDP binding (nmol/mg protein)	8	0.088 ± 0.014	0.143 ± 0.026	NS
[^{3}H] ADP binding (nmol/mg protein	8	0.068 ± 0.011	0.116 ± 0.023	NS
Proportion of total polypeptides in 25 000 - 35 000 region	9	20.86 ± 0.63	20.22 ± 0.42	NS

Note: Body weights shown are those before the first injection (Start) and at the time of the experiment, 2 weeks later (Finish). Data shown under IBAT refer to the whole tissue or to homogenates of the tissue. Data shown under IBAT mitochondria refer to isolated mitochondria. Values are means ± SE.

* NS, not significant.

increase in tissue size, total protein and cytochrome oxidase content than when NE is administered alone (Fig. 64 and Table VIII), but there is no difference in the extent of binding of purine nucleotides to mitochondria of treated or untreated rats. The tissue hypertrophy induced by hormone treatment is however smaller than that induced by cold exposure (Table VIII). Possibly, the injections of a hormone with a short half-life, such as NE, may not mimic perfectly the effect of cold, when NE is secreted at a high rate on a continuous basis. It is possible that the tissue hypertrophy induced by long term cold exposure may be mediated by NE. Again, an indirect action of NE can not be excluded from these experiments. It is not known whether NE can stimulate the differentiation of some brown fat precursor cells. NE itself does not stimulate the rate of protein synthesis in isolated adipocytes (33). Nevertheless, the changes in mitochondrial composition which result in an increased concentration of proton conductance pathway sites are not mediated by NE.

Conclusions: NE is most probably responsible for the unmasking of proton conductance pathway sites during the acute phase of exposure to cold but is not responsible for the increase in concentration of these sites occurring during the adaptive phase of cold acclimation. The hormone(s) inducing the increased synthesis of proton conductance pathways is (are) still unknown.

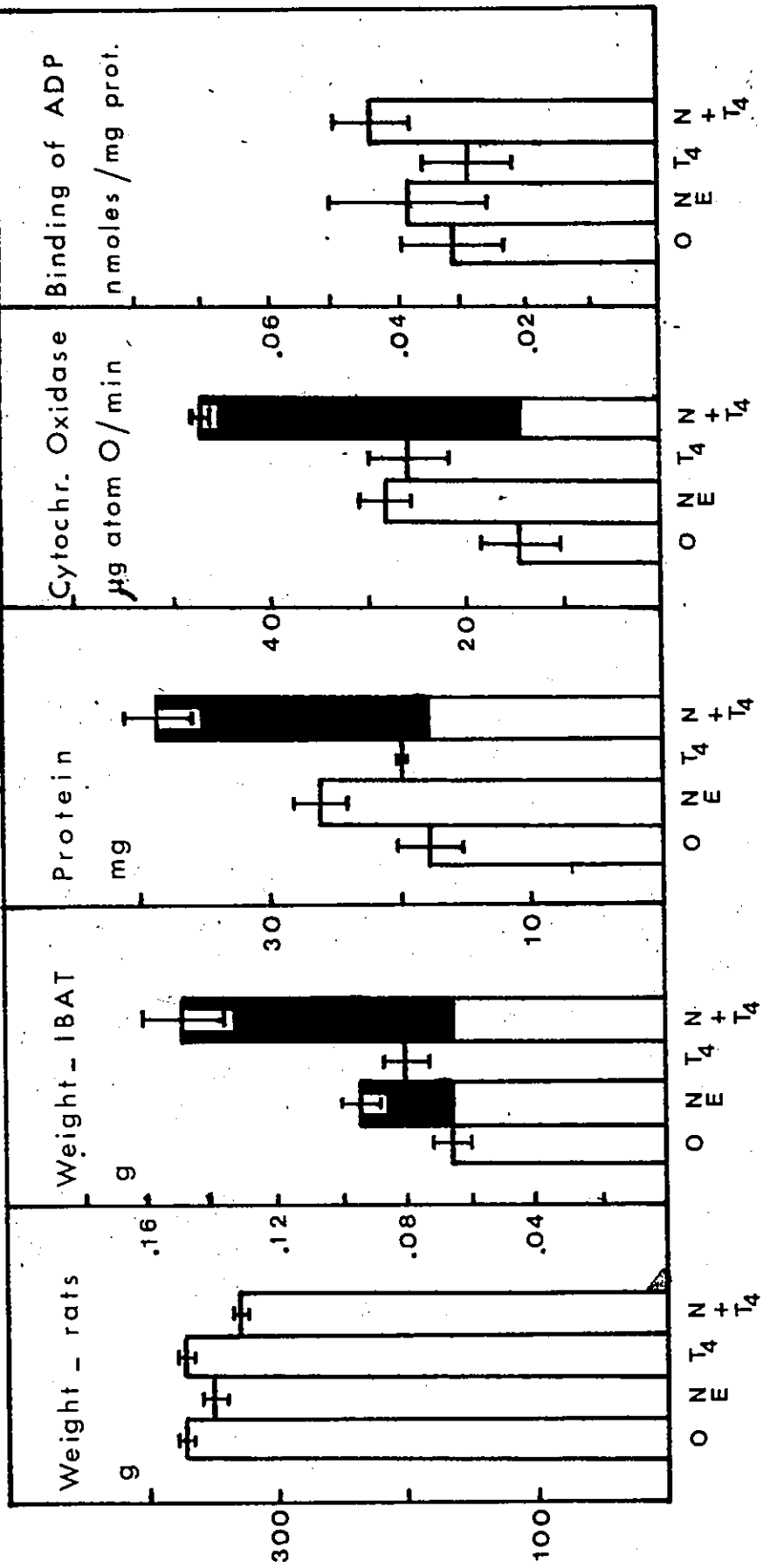


FIG. 64. Effect of long-term treatment (3 weeks) with norepinephrine (NE), thyroxine (T_4) or a combination of both (N + T_4) in oil on brown adipose tissue.

Rats receiving only peanut oil served as controls (O). Total protein and cytochrome oxidase contents were measured on tissue homogenates. Binding of ^{3}H-ADP was estimated on isolated BAT mitochondria. Values are the means ± S.E. of 3 separate experiments.

TABLE VIII. Chronic effects of cold, norepinephrine (NE), thyroxine (T_4) or combination of NE and T_4 on the interscapular brown adipose tissue of warm-acclimated rats

	WARM	COLD	W-OIL	W-NE	W-T_4	W-NE + T_4
Body weights (g)						
Start	237 ± 6	232 ± 6	232 ± 8	239 ± 9	225 ± 5	221 ± 7
Finish	367 ± 9	319 ± 7 *	369 ± 7	351 ± 11	368 ± 5	329 ± 7 *
IBAT						
Wet weight (g/100 g, B.W.)	0.07 ± .00	.23 ± .02*	.07 ± .00	.10 ± .01*	.08 ± .01	.15 ± .01*
Total protein (mg)	16 ± 1	78 ± 3 *	18 ± 2	27 ± 2	20 ± 1	39 ± 2 *
Total cytochrome oxidase (µg atoms O/min)	16 ± 3	161 ± 49 *	15 ± 4	28 ± 3	26 ± 4	47 ± 1 *
Specific activity of cyto. oxid. (µg atoms O/min-mg protein)	1.0 ± .2	2.1 ± .6	0.9 ± .3	1.1 ± .1	1.3 ± .2	1.7 ± .5
IBAT mitochondria						
^{3}H-GDP binding (nmoles/mg protein)	0.02 ± .00	.19 ± .01*	.03 ± .01	.02 ± .00	.02 ± .01	.03 ± .00
^{3}H-ADP binding (nmoles/mg protein)	0.03 ± .01	.26 ± .01*	.03 ± .01	.04 ± .01	.03 ± .01	.04 ± .01

Note: Body weights shown are those before the first injection (Start) and at the time of the experiment, 3 weeks later (Finish). Data shown under IBAT refer to the whole tissue or to homogenates of the tissue. Data shown under IBAT mitochondria refer to isolated mitochondria. Values are means ± SE of 3 experiments.

* = $p < 0.05$ when compared to warm-acclimated untreated controls.

W-A rats were injected with norepinephrine (NE), thyroxine (T_4) or combination of both (NE + T_4) in oil for 3 weeks. Rats receiving only peanut oil served as controls (OIL). For comparison, rats were left in the warm (WARM) or in the cold (COLD) without any treatment for 3 weeks.

C. ATTEMPTS TO PRODUCE UNMASKING OF PURINE NUCLEOTIDE BINDING SITES IN VITRO IN ISOLATED BROWN ADIPOSE TISSUE MITOCHONDRIA FROM WARM-ACCLIMATED RATS

Unmasking of purine nucleotide binding sites takes place during the period of acute cold stress and upon NE intravenous infusion (see Section II, A-1 and Section III, B-1). At the same time, mitochondria appear larger and their internal structure is altered (see Section II, A-2). Unmasking does not depend on protein synthesis (see Section III, A) and is believed to reflect a chemical or physical transformation of mitochondrial composition or structure. The changes are maintained throughout the mitochondrial isolation procedure and may then be considered of a "permanent" nature in vitro. If the mechanism of unmasking involves a reorganization of mitochondrial structure, it may be possible to reproduce it in vitro by altering the conformation of isolated mitochondria. Several factors are known to affect mitochondrial morphology and their effects on purine nucleotide binding of isolated mitochondria from young warm-acclimated rats are described in this section in attempt to gain some insight about the mechanism by which unmasking might occur in vivo.

Objective: The purpose of this work is to attempt to produce changes in vitro similar to these occurring upon acute cold stress or NE infusion in order to elucidate the mechanism by which these changes may arise.

1. Effect of matrix swelling on purine nucleotide binding by mitochondria isolated from young warm-acclimated rats

Isolated mitochondria from rats acutely exposed to cold are larger as measured by the Coulter Counter (Fig. 31). Only overall mitochondrial volume can be detected by the Coulter Counter and not the conformational state of the inner membrane (246). Enlargement of mitochondria may be due to large amplitude swelling within the tissue although the matrix of the sucrose-isolated mitochondria is highly condensed (Fig. 15, 16). Larger mitochondria have been reported in BAT sections from cold-exposed rats (270, 271, 279) and a reversible mitochondrial swelling in tissue sections from newborn rats after administration of NE (288). It is known that various morphological changes are possible during swelling which appears to vary with cell type and with the nature of the swelling agents (177).

Objective: The purpose of this work is to find out whether matrix expansion has an effect on purine nucleotide binding properties and might be held responsible for unmasking of sites occurring during acute cold exposure and NE infusion.

Method: Mitochondria from 6 W-A rats weighing 170-250 g were isolated as usual and resuspended in the isolation medium (SHE) at a concentration of 13-17 mg/ml. Then, 0.2 ml of mitochondrial suspension was added to 5.3 ml incubation medium I, II or III. Incubation medium I contained 100 mM sucrose, 10 mM choline chloride, 1mM EDTA (disodium salt), 20 mM TES and 5 μM rotenone at pH 7.1. Incubation medium II contained 100 mM KCl, 1 mM EDTA (disodium salt), 20 mM TES and 5 μM rotenone at pH 7.1.

Incubation medium III contained the same constituents as medium II plus 0.5 μM valinomycin. Mitochondria were incubated at room temperature for 20 minutes. At various time intervals, (1, 3, 6, 10, 20 minutes) 0.5 ml aliquots were taken and transferred to microcentrifuge tubes (Eppendorf). The tubes already contained incubation medium I, II or III supplemented with ^{14}C-sucrose (0.1 μCi/ml) and 100 μM potassium atractyloside. Final volume was 0.75 ml and purine nucleotide binding was measured as described previously. ADP (100 μM) was used to displace bound tritiated nucleotides (^{3}H-GDP).

Mitochondrial swelling was followed by observing the decrease in optical density at 540 nm as a function of time of a mitochondrial suspension (0.7 - 0.9 mg/ml) with a Gilford spectrophotometer model 2400-2. Measurements of changes in the absorption of light in the visible region by a suspension of mitochondria is a measure of mitochondrial volume which is simple, sensitive and qualitatively reliable (177). It is an indirect measure of light scattering that has been successfully applied to liver (276, 277) and BAT mitochondria (204). Light absorption by mitochondria depends on two factors: mitochondrial volume and the refractive index difference between the suspending medium and the mitochondria.

Results and discussion: Very little swelling occurs during the 20 minute incubation period when mitochondria are suspended in sucrose medium (I) as indicated by the absence of decrease in optical density (O.D.) of the mitochondrial suspension (Fig. 65). Water uptake occurs when mitochondria are suspended in KCl medium (II and III). Swelling occurs at a faster rate in presence of valinomycin (a K^+ ionophore) although the extent of matrix expansion remains the same. However, very

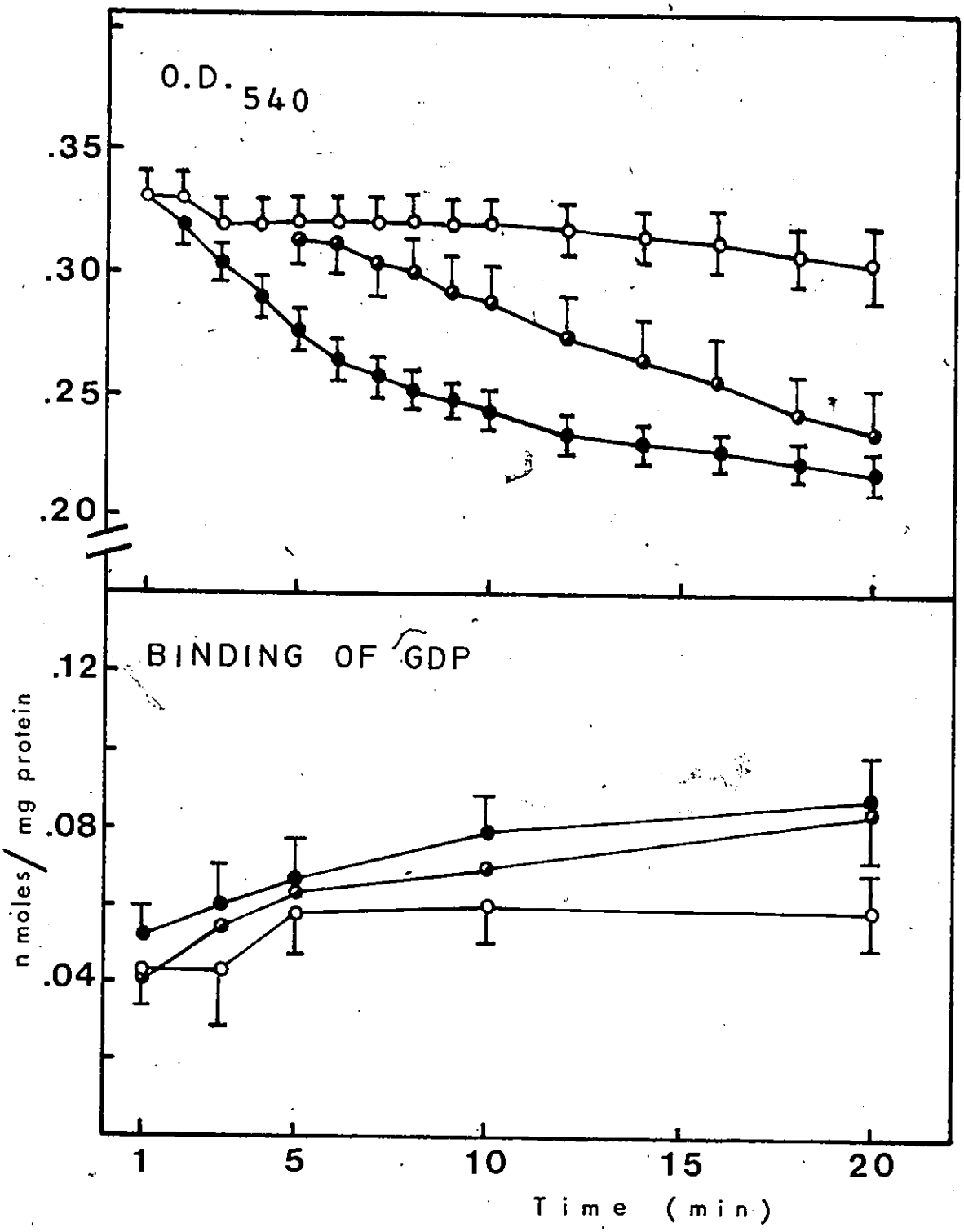


FIG. 65. Effect of swelling on GDP binding of isolated BAT mitochondria from young W-A rats.

Mitochondria were incubated in sucrose medium (I) (o—o), or in KCl medium with (III) (●—●) or without (II) (●—○) valinomycin. Swelling is indicated by a decrease in optical density at 540 nm ($O.D._{540}$). No significant difference is observed in GDP binding at any time. Values are the means ± S.E. of 3 separate experiments.

little change is observed in purine nucleotide binding by mitochondria during swelling.

Conclusions: Matrix expansion due to swelling is not responsible for unmasking of purine nucleotide binding sites in BAT mitochondria.

2. Effects of addition or removal of fatty acids on purine nucleotide binding of isolated mitochondria from warm-acclimated rats

It is known that the morphology of BAT mitochondria is different in presence and absence of bound fatty acids (28). Mitochondria incubated in a medium containing ATP, CoASH and carnitine have an orthodox conformation with a large expanded matrix and very small intracristal space (intermembrane space). On the other hand, mitochondria incubated in the presence of palmitate have a very condensed matrix and large intracristal space. In this thesis, freshly isolated mitochondria from BAT have the latter appearance. Indeed, mitochondria isolated from BAT are known to have a large amount of fatty acids bound to them (98, 112, 213).

Fatty acids are also known to cause a large increase in the proton conductance of BAT mitochondrial inner membrane (112) and respiratory control can be re-established by their removal (31, 81, 112, 136, 211). Fatty acyl CoA esters are known to displace allosterically purine nucleotides bound to BAT mitochondria (35, 112) and have been proposed as possible physiological mediators of NE action (195). Since the tissue fatty acid content varies during the period of cold acclimation, it was then of interest to study the effects of addition or removal of fatty acids on purine nucleotide binding of isolated BAT mitochondria.

Objective: The purpose of this work was to find out whether the addition or removal of fatty acids has an unmasking effect in isolated mitochondria from BAT of young warm-acclimated rats.

Method: Mitochondria were isolated from IBAT of 4-6 W-A rats (236 ± 15 g) and resuspended in 1.0 ml isolation medium (SHE) at a protein concentration of 10-15 mg/ml.

For studying the effect on purine nucleotide binding of a pre-incubation with fatty acyl CoA esters, 0.2 ml mitochondrial suspension was added to 5.0 ml incubation medium containing 100 mM KCl, 1mM EDTA (disodium salt), 20 mM TES, 5 µM rotenone and palmitoyl CoA at a final concentration ranging from 0 to 10 µM. The pH of the incubation medium was 7.1 and the incubation was done at room temperature for 20 minutes. At the end of the incubation period, the mitochondrial suspension was diluted with 35-40 ml of cold isolation medium (SHE) and centrifuged at 16 000 xg for 15 minutes. Mitochondria were finally resuspended in isolation medium (SHE) and the GDP binding assay performed as described previously. A total of 12 warm-acclimated rats were used for this experiment.

Bound fatty acids were removed from mitochondria by two different methods. In the first method, 0.2 ml mitochondrial suspension was added to 5.0 ml incubation medium containing 100 mM KCl, 1 mM EDTA, 20 mM TES, 5 µM rotenone and 4% (w/v) albumin (fatty acid free) at pH 7.1. The incubation was performed at room temperature for 20 minutes. A parallel incubation in which the medium did not contain any albumin served as control. In the second method, 0.2 ml mitochondrial suspension was added to 3.0 ml incubation medium in a Yellow Springs Instruments Oxygen monitor

chamber thermoregulated at 25°C. The incubation medium contained 100 mM KCl, 3 mM l-malate (Na salt), 20 mM TES, 2mM $MgCl_2$, 1 mM EDTA disodium salt), 4 mM KH_2PO_4, 5 µM CoA and 1.8 mM ATP at pH 7.2. Mitochondria were incubated for 2-3 minutes before the addition of 50 µl of D-L carnitine (final concentration 3.6 mM). Mitochondrial respiratory rates were recorded before and after addition of carnitine. The oxygen content of the incubation medium was assumed to be 490 nmoles O_2/ml. A typical experiment and the respiratory rates obtained from 3 separate experiments are illustrated in Fig. 66.

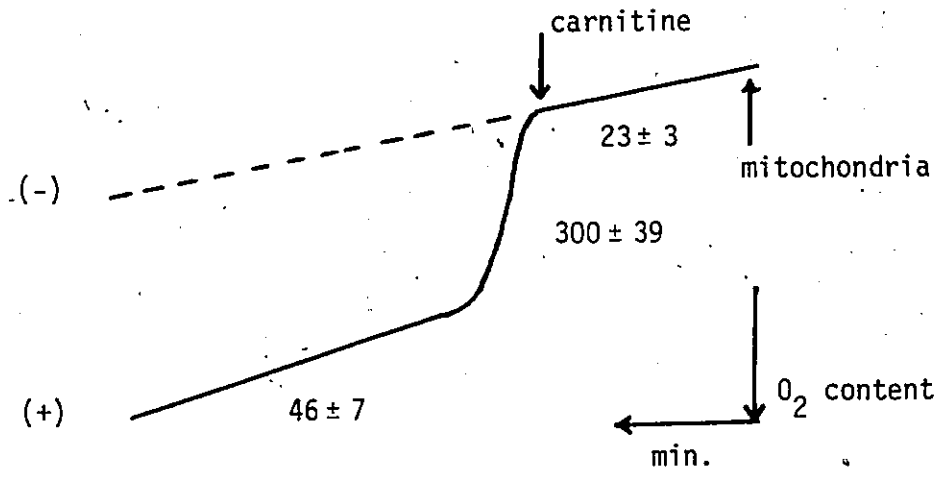


FIG. 66. Respiratory rates of BAT mitochondria from W-A rats before and after addition of DL-carnitine. Respiratory rates are expressed in nmoles O_2/min-mg protein and are means ± S.E. of 3 experiments.

Total incubation period was 20 minutes. A parallel incubation in which no carnitine was added served as control.

At the end of the incubation periods for both methods, mitochondrial suspensions were diluted with 35-40 ml cold isolation medium (SHE), cen-

trifuged at 16 000 x g for 16 minutes and finally resuspended in isolation medium (SHE). GDP binding was measured as described previously. A total of 13 warm-acclimated rats was used for this experiment.

Results and discussion: A pre-incubation of BAT mitochondria isolated from W-A rats with increasing concentration of palmitoyl CoA reduces the extent of GDP binding (Fig. 67). However, the concentration of palmitoyl CoA needed to obtain a significant reduction is very high (10 μM) which corresponds to 20-30 nmoles added palmitoyl CoA per mg mitochondrial protein. Upon isolation of BAT mitochondria, there is usually 3-5 nmoles bound palmitoyl CoA per mg protein (32). Direct displacement of bound tritiated GDP from BAT mitochondria by similar concentration of palmitoyl CoA has been reported (35). These results may be important to consider as regard to the tissue homogenization conditions in which tissue fat content may vary. Very low binding data for mitochondria from W-A rats may be due to the large amount of fatty acids bound to them.

Indeed, removal of fatty acids by albumin gives rise to an increase in GDP binding from 0.090 ± .017 nmoles/mg protein to 0.151 ± .014 nmoles GDP bound/mg protein ($p < 0.05$) (Fig. 68, B). The increase thus obtained is however much lower than that observed after NE infusion or early cold exposure (see Fig. 26, 60, 61).

Unexpectedly, removal of fatty acids by carnitine treatment induces a decrease in purine nucleotide binding (Fig. 68, A). All the endogenous content of fatty acids has been oxidized as indicated by the low mitochondrial respiratory rate obtained 20 minutes after the carnitine addition (Fig. 66). The opposite effect on GDP binding of removal of

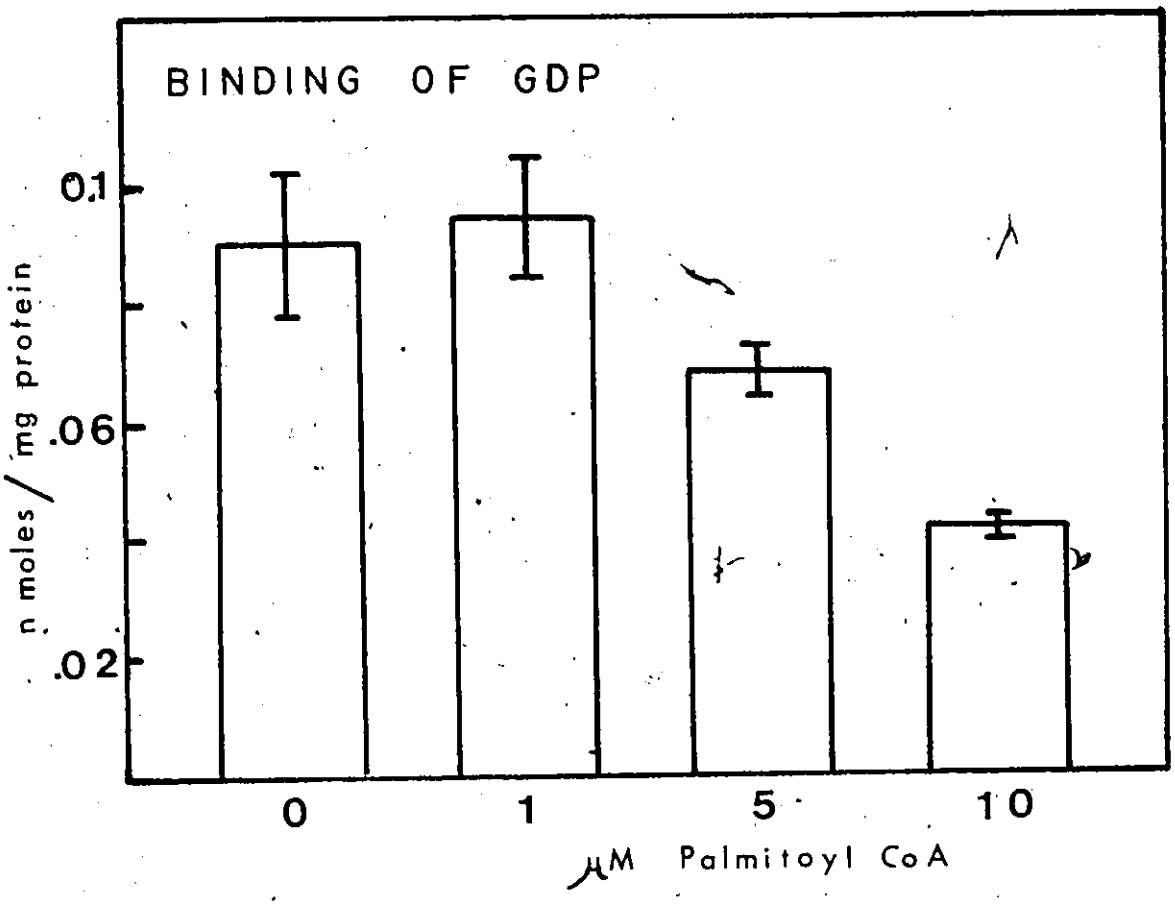


FIG. 67. Effect of palmitoyl CoA pre-incubation on ^{3}H-GDP binding of BAT mitochondria of W-A rats.

Mitochondria were incubated in KCl-medium supplemented with various concentrations of palmitoyl CoA and re-isolated in sucrose medium as described in methods. Values are the means ± SE of 3 separate experiments.

The GDP binding data are:

Palmitoyl CoA (μM)	GDP binding (nmoles/mg protein)	
0	0.090 ± 0.012	
1	0.094 ± 0.010	(N.S.)
5	0.068 ± 0.006	(N.S.)
10	0.042 ± 0.002	(p 0.02)

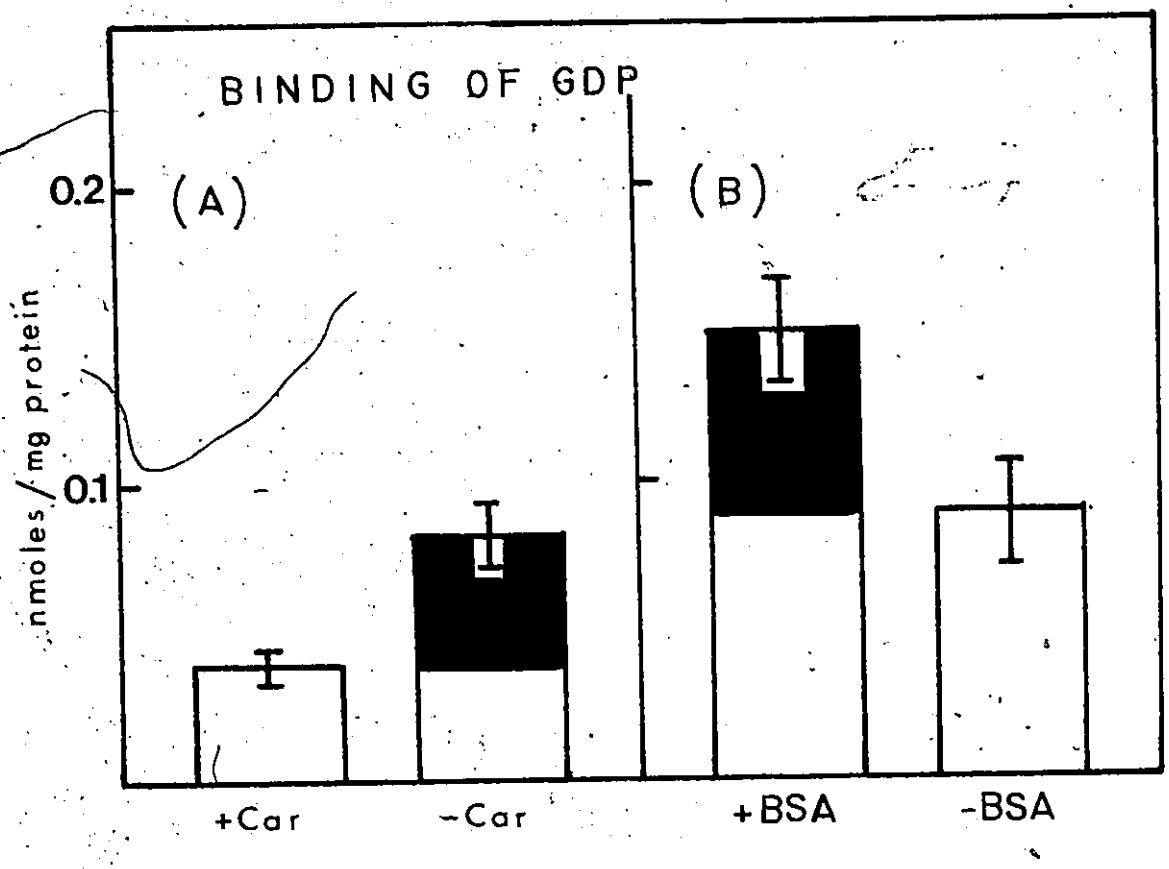


FIG. 68. Effect of removal of bound fatty acids on ^{3}H-GDP binding of BAT mitochondria from W-A rats.

In part A, mitochondria were incubated in presence (+Car) or absence of carnitine (-Car) and thereafter re-isolated (see Methods). The binding data are: + Car. 0.039 ± .006 nmoles/mg protein and -Car 0.082 ± .011 nmoles/mg protein ($n = 3$, $p < .05$).

In part B, mitochondria were incubated in presence (+BSA) or absence (-BSA) of albumin (4% w/v) and thereafter re-isolated. The binding data are: + BSA 0.151 ± .014 nmoles/mg protein, -BSA 0.089 ± 0.017 nmoles/mg protein ($n=3$, $p < .05$). Values are means ± S.E.

bound fatty acids by the two methods may probably be ascribed to the completeness of removal of bound fatty acids from mitochondria. Albumin action may be ascribed to the removal of bulk fatty acids bound to the outer face of the inner mitochondrial membrane known to have an allosteric displacing effect on purine nucleotides (35, 112). Albumin is however unable to remove the totality of bound fatty acids. Carnitine treatment removes a larger fraction of fatty acids from the mitochondria. It is known that fatty acid content is reduced from 17 nmoles palmitate/mg protein to 1 nmole/mg protein in hamster mitochondria incubated with CoA, ATP and carnitine (112). About 80-85% endogenous fatty acids are removed by albumin (112). Carnitine treated mitochondria are known to be fully recoupled and they have a largely expanded matrix and very little intracristal space (28). The degree of recoupling of mitochondria depends on the amount of bound fatty acids removed (112). The difference in purine nucleotide binding of mitochondria in which bound fatty acids were removed by carnitine or albumin treatment is probably due to a difference in mitochondrial morphology resulting from the degree of removal of fatty acids.

Another possibility is the formation of acyl CoA from bound fatty acids by the carnitine-CoA-ATP treatment. It was shown in Fig. 67 that the presence of extramitochondrial fatty acyl CoA decreases the purine binding to BAT mitochondria. The amount of extramitochondrial acyl CoA required is very high (20-30 nmoles/mg mitochondrial protein) but it is possible that the production of acyl CoA in the immediate vicinity of the inner mitochondrial membrane induces a concentration gradient that may account for the reduced GDP binding.

Conclusions: Although the extent of binding of purine nucleotides to BAT mitochondria is altered by addition or removal of fatty acids, the magnitude of the changes observed is too low to account for the unmasking of proton conductance pathways occurring during acute cold exposure and NE infusion.

3. Effect of temperature and pH on purine nucleotide binding of BAT mitochondria

It is known that the pH of the incubation medium is an important factor to consider when studying the energy conservation properties of isolated BAT mitochondria.

In the absence of albumin, with or without purine nucleotides, the respiratory rates of isolated hamster BAT mitochondria decrease as the pH of the incubation medium increases (200). In the presence of nucleotides, P/O ratios and respiratory control decrease as the pH of the incubation medium increases (99).

In the presence of albumin, with or without purine nucleotides, the respiratory rates of isolated hamster BAT mitochondria increase as the pH of the incubation medium increases (200). In presence of nucleotides, respiratory control decreases as the pH of the incubation medium increases (200).

These contradictory effects of pH on mitochondrial respiration may be due to several interlinked factors. For instance, it is known that the binding of purine nucleotides controlling the proton conductance pathway of BAT mitochondria is pH dependent; the affinity of purine nucleotides decreases as the pH of the incubation medium increases (196).

The pH optimum of various mitochondrial respiratory enzymes and substrate dehydrogenases is certainly also a factor to consider as well as the dissociation constants of some important compounds. For example, bound fatty acids or fatty acyl CoA possess pK_a values of 7.0 - 7.2 in an hydrophobic environment (32, 81).

The respiratory rate of brown adipocytes is also affected by the pH of the incubation medium. They have a slightly higher basal respiratory rate when incubated at pH 7.4 than at pH 6.9 (192). It has also been suggested from indirect calorimetry experiments that an intracellular alkalinization may trigger mitochondrial energy dissipation in brown adipocytes (42).

Objective: The objective of this work was to investigate the effect of pH as possible means of unmasking purine nucleotide binding sites in BAT mitochondria from warm-acclimated rats.

Method: Mitochondria from 6 young (4-5 weeks old) W-A rats weighing 240 ± 5 g were isolated as usual and resuspended in isolation medium (SHE) at a protein concentration of 13-16 mg/ml. Then, 0.2 ml mitochondrial suspension was added to 5.0 ml incubation medium containing 100 mM KCl, 20 mM TES, 1 mM EDTA (disodium salt) and 5 µM rotenone. The pH of the incubation medium was varied from 6.7 to 7.9 by the addition of KOH (1N) and the incubation performed at 3 different temperatures (4, 23, 37°C) for 20 minutes. At the end of the incubation period, the mitochondrial suspensions were diluted with 35-40 ml cold isolation medium (SHE), centrifuged at 16 000 x g for 15 minutes and finally resuspended in isolation medium (SHE). Measurements of purine nucleotide binding and

size distribution as well as electron microscopy were performed as described previously. A total of 32 rats was used for these experiments.

The effect of age and cold acclimation on the unmasking action of pH was also studied. For this purpose, mitochondria were isolated from 4 W-A rats (8–10 weeks old) weighing 459 ± 21 g and 1 C-A rat (8-10 weeks old of which 5 have been spent at 4°C) weighing 363 ± 6 g and the experiment performed as described above. A total of 12 W-A and 3 C-A rats have been used.

Results and discussion: Figure 69 illustrates the effect of pH and temperature during pre-incubation on subsequent purine nucleotide binding of mitochondria from young W-A rats. A large increase in GDP binding is observed at alkaline pH. The changes are temperature dependent. No effect of pH is observed at 4°C while maximum GDP binding occurs at higher pH at room temperature than at 37°C. The maximum purine nucleotide binding value obtained is the same as that observed after NE infusion or acute cold exposure (see Fig. 26, 61). Mitochondria have been sucrose re-isolated after their incubation at various pH and the effect observed is then very different from the direct purine nucleotide binding pH dependency observed by Nicholls (196). In fact, in these experiments, mitochondria retained a chemical or structural transformation induced by alkaline pH.

Figures 70-73 show electron micrographs of BAT mitochondria that had been pre-incubated in KCl medium at room temperature and pH varying from 6.7 to 7.9 and thereafter re-isolated. Swollen mitochondria characterized by an expanded matrix space and very little intracristal space are observed on Figures 70 and 71. These mitochondria had been

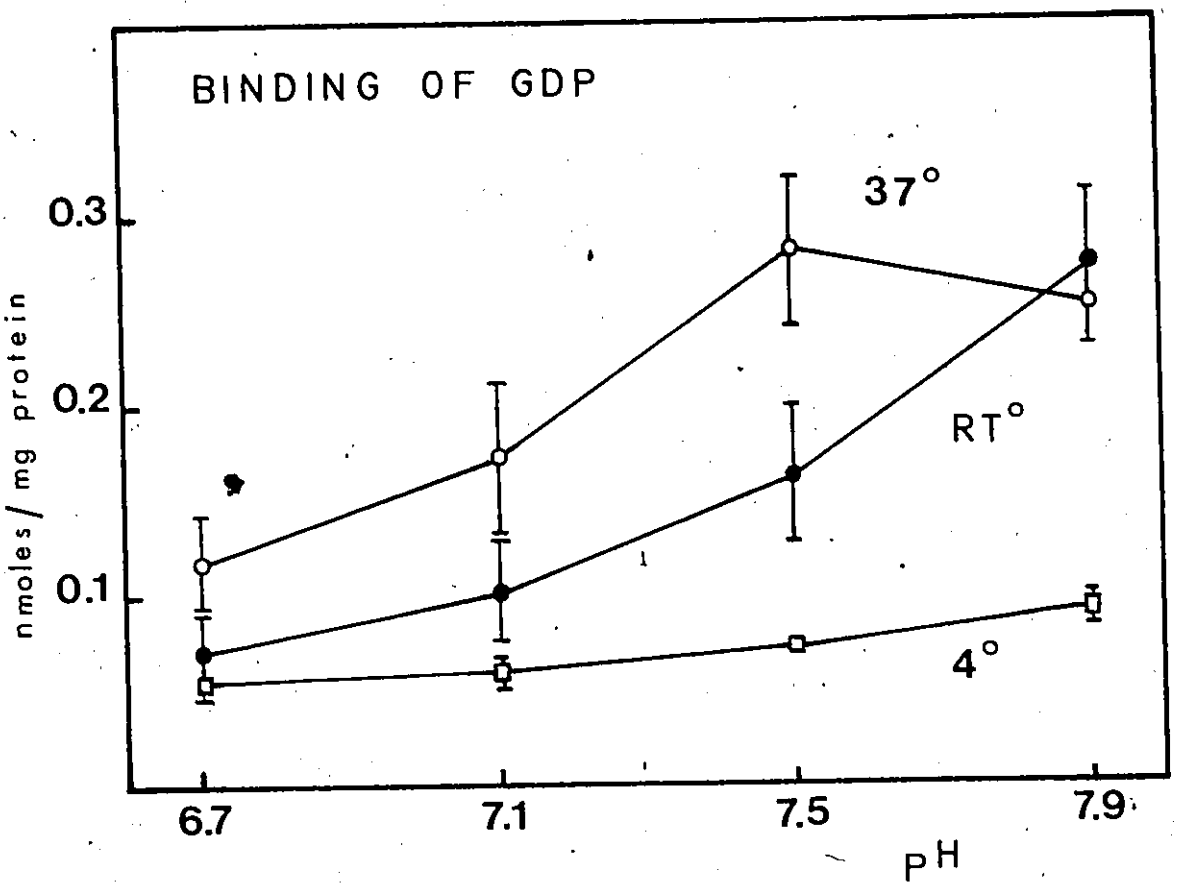


FIG. 69. Effect on ^{3}H-GDP binding of a pre-incubation of mitochondria from IBAT of young (4-5 weeks old) W-A rats at various pH.

Mitochondria were incubated at different temperatures in KCl-medium (see Methods) at various pH for 20 minutes and thereafter re-isolated. Binding of ^{3}H-GDP was measured as usual at pH 7.1 Values are the means ± SE of 3 separate experiments.

The actual binding data are: (nmoles/mg protein)

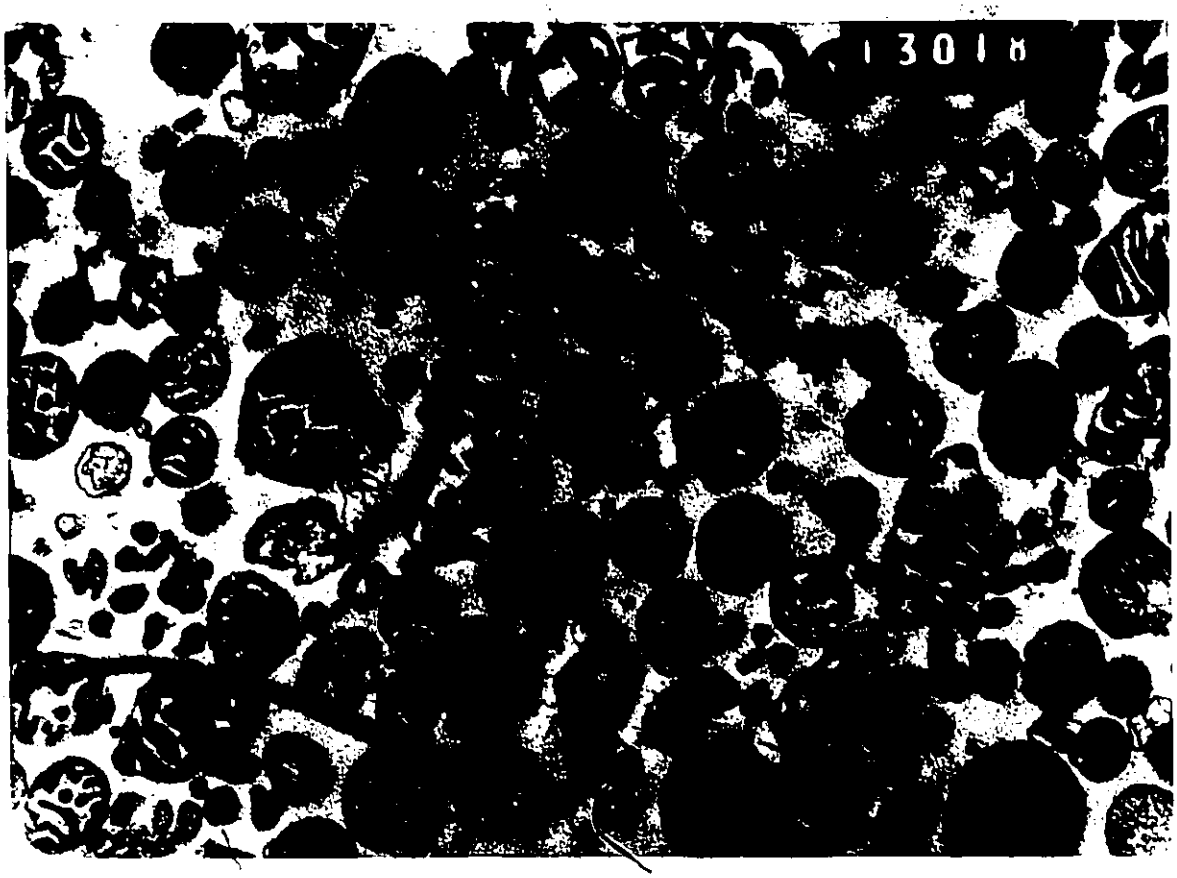
pH/T°	4°C	RT°	37°C
6.7	0.056 ± .004	0.073 ± .022	0.121 ± .023
7.1	0.059 ± .007	0.102 ± .029	0.176 ± .038*
7.5	0.072 ± .004†	0.163 ± .037	0.285 ± .042* †
7.9	0.090 ± .006†	0.277 ± .021* †	0.255 ± .041* †

Note: * $p < .05$ or better when compared with data at 4°C
† $p < .05$ or better when compared with data at pH 6.7

FIG. 70. Isolated IBAT mitochondria from young (4-5 weeks old) W-A rats pre-incubated in KCl-medium at pH 6.7 and thereafter sucrose re-isolated.
(x 16 000)

FIG. 71. Isolated IBAT mitochondria from young (4-5 weeks old) W-A rats pre-incubated in KCl-medium at pH 7.1 and thereafter sucrose re-isolated.
(x 16 000)

70



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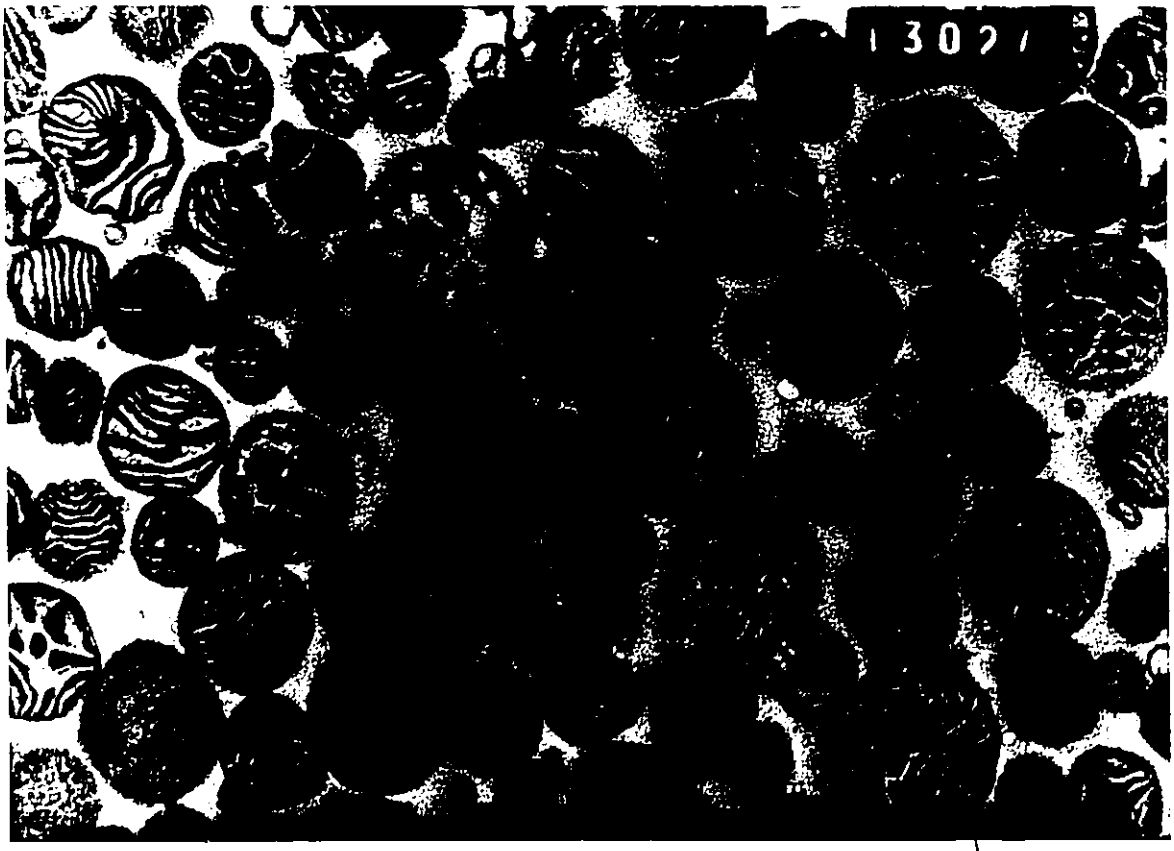
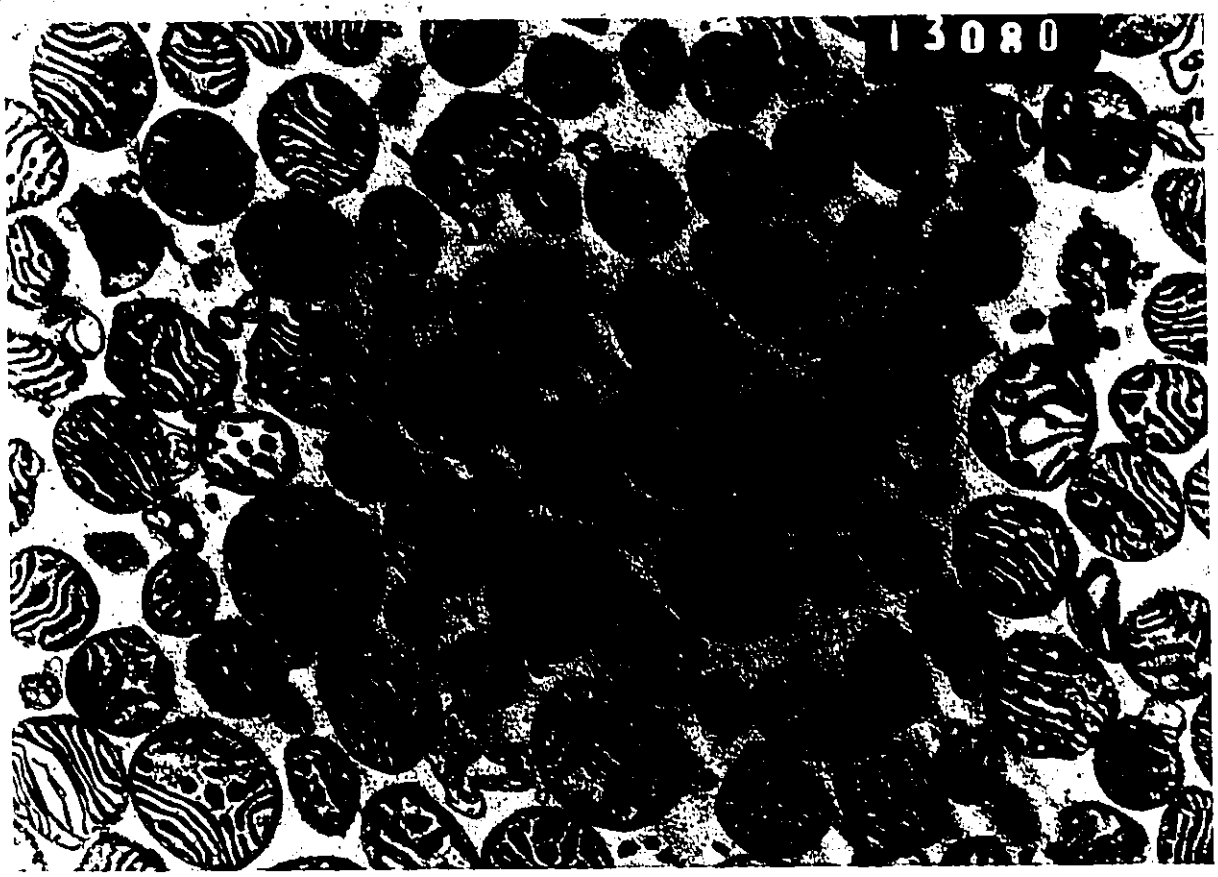


FIG. 72. Isolated IBAT mitochondria from young (4-5 weeks old) W-A rats pre-incubated in KCl-medium at pH 7.5 and thereafter sucrose re-isolated.
(x 16 000)

FIG. 73. Isolated IBAT mitochondria from young (4-5 weeks old) W-A rats pre-incubated in KCl-medium at pH 7.9 and thereafter sucrose re-isolated.
(x 16 000)

72



73

pre-incubated at low pH (6.7 and 7.1). Most mitochondria still have the "dotted" internal structure although in a swollen state at pH 6.7 and 7.1. Treatment at pH 6.7 is detrimental to the mitochondria where many of them appear broken. At alkaline pH (7.5 and 7.9) (Fig 72, 73) there is an increase in the number of mitochondria with a parallel arrangement of cristae and fewer swollen mitochondria are observed. At pH 7.9, mitochondria have a very condensed matrix and large intracristal space assuming "honeycomb or coalescent" conformation (Fig. 73). The disappearance of mitochondria with "dotted" internal structure is to be remarked.

Mitochondria pre-incubated at pH 7.9 have also their size distribution curve shifted toward mitochondria of smaller sizes when compared with the size distribution of mitochondria pre-incubated at pH 6.7 (Fig. 74 and Table IX). There is a larger number of mitochondria with a volume smaller than 0.2 μm^3. Mitochondria pre-incubated at pH 7.1 and 7.5 have an intermediate distribution between these obtained at pH 6.7 and 7.9. These results stress again the lack of correlation between mitochondrial size or swelling of matrix and the unmasking of purine nucleotide binding sites.

The effect of pH on purine nucleotide binding is probably best explained in two ways. The large increase in GDP binding at alkaline pH probably results from a reorganization of the inner membrane structure from a "dotted" to a more parallel arrangement of cristae increasing the membrane surface and unmasking the sites. Secondly, there is probably a greater accessibility of exogenous GDP to the binding sites at alkaline pH because of the larger intracristal intermembrane space present in these mitochondria. The pH induced unmasking of purine nucleotide binding sites

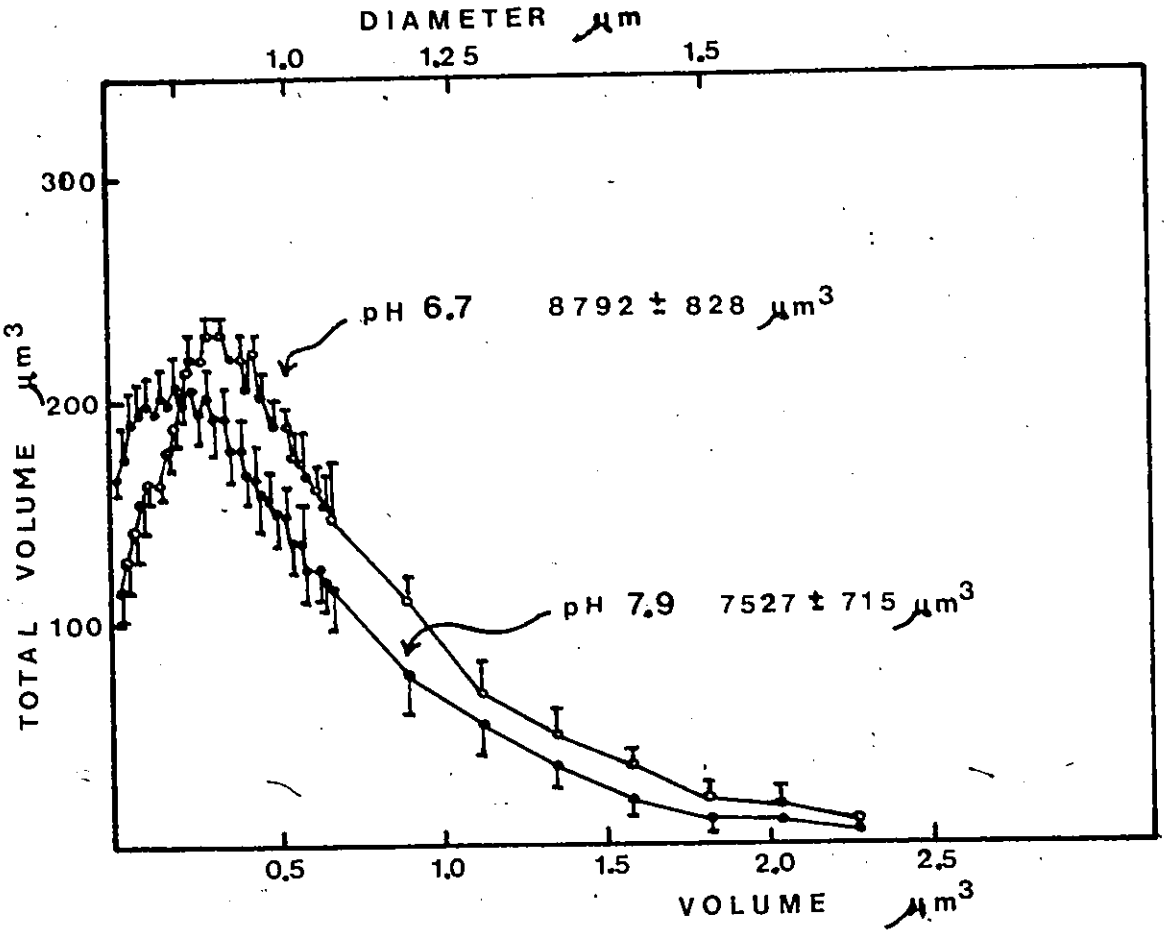


FIG. 74. Effect of pre-incubation at various pH on mitochondrial size distribution of mitochondria isolated from IBAT of young (4-5 weeks old) W-A rats.

Mitochondria were incubated at room temperature in KCl-medium (see Methods) at various pH and thereafter re-isolated in sucrose medium. This figure shows the total volume contributed by mitochondria of sizes from 0.0217 μm^3 to 2.18 μm^3. Volumes shown are the means ± SE of 3 separate experiments. When no error standard is shown, the error is too small to be shown separately from the point indicating the mean.

TABLE IX. Percentage of total volume contributed by mitochondria of sizes smaller than 0.2 μm^3.

IBAT mitochondria from W-A or C-A rats were incubated at room temperature in KCl medium (see Methods) at various pH and thereafter re-isolated in sucrose medium. Results shown are means ± SE of three separate experiments.

pH	6.7	7.1	7.5	7.9
W-A (4 - 5 weeks old)	16.9± 0.6	17.0± 0.7	19.4± 0.9	24.4± 1.8
P <		N.S.	N.S.	.02
W-A (8-10 weeks old)	13.3± 0.3			24.1± 1.7
P <				.005
C-A (8-10 weeks old)	15.8± 1.6			16.4± 0.7
P <				N.S.

Note: The statistical significance is calculated relative to the results obtained at pH 6.7

C-A had been acclimated to cold (4°C) for 5 weeks.

of mitochondria from older warm-acclimated rats still occurs but the total number of sites that can be unmasked appears lower (Fig. 75). A pH dependent increase in GDP binding also occurs in BAT mitochondria from C-A rats (Fig. 75). However, the extent of GDP binding by mitochondria pre-incubated at pH 6.7 is much lower than normally obtained with sucrose isolated mitochondria from C-A rats (compare Fig. 47 and 75). The maximum GDP binding value obtained is also not higher than normally obtained with mitochondria from C-A rats. It is concluded that no actual unmasking of sites is occurring in mitochondria from C-A rats but rather a reduction of binding at low pH most probably resulting from the inaccessibility of binding sites to exogenous purine nucleotides because of large matrix expansion and subsequent reduction in intracristal space. Expansion of matrix of mitochondria from hamster BAT is known to occur in hypotonic sucrose or in solution of permeable salts (32). On the other hand, the GDP binding value obtained after pre-incubation of mitochondria from W-A rats (particularly with young animals) at alkaline pH is much higher than normally obtained after sucrose isolation and corresponds to these observed after acute cold exposure or NE infusion.

Conclusions: Unmasking of purine nucleotide binding sites in warm-acclimated rats is probably mediated by a pH-dependent reorganization of mitochondrial membranes. The actual mechanism by which alkaline pH induces the mitochondrial transformation is unknown. These results however support the hypothesis of Chinet and co-workers (42) that an intracellular alkalinization induced by NE may trigger energy dissipation in mitochondria of brown adipocytes.

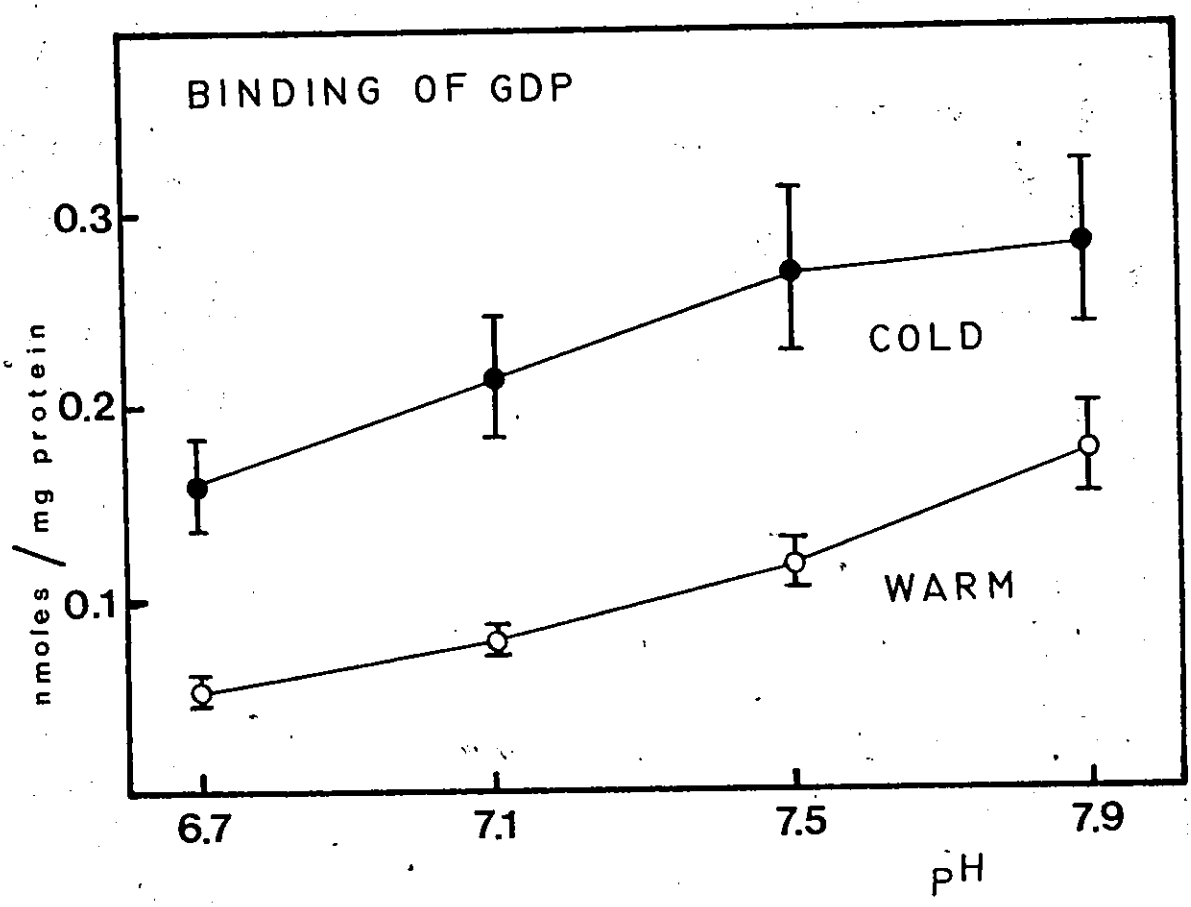


FIG. 75. Effect on ^{3}H-GDP binding of a pre-incubation at various pH of mitochondria from W-A and C-A rats.

The animals are 8-10 weeks old of which the last 5 weeks were spent at 4°C for the C-A rats. Mitochondria were incubated at room temperature in KCl-medium (see Methods) at various pH for 20 minutes and thereafter re-isolated in sucrose medium. Binding of ^{3}H-GDP was measured as usual at pH 7.1. Values are means ± SE of 3 separate experiments.

The actual binding data are:(nmoles / mg protein)

pH	6.7	7.1	7.5	7.9
W-A	0.053 ± .006	0.078 ± .007	0.116 ± .012†	0.172 ± .022†
C-A	0.158 ± .023*	0.211 ± .032*	0.266 ± .043*	0.281 ± .041

Note: * $p < .05$ or better when compared with similarly treated mitochondria from W.A rats.

† $p < .05$ or better when compared with mitochondria from the same group incubated at pH 6.7.

GENERAL DISCUSSION AND CONCLUSIONS

The main finding described in this thesis is that adaptive changes in the proton conductance pathway of brown adipose tissue mitochondria are associated with the altered capacity for cold-induced nonshivering thermogenesis in the cold-acclimated rat. The biphasic nature of the cold-induced mitochondrial changes is stressed. The findings lead to further questions about the mechanisms underlying the changes and suggest additional lines of investigation, as outlined below, to elucidate the mechanisms associated with the rapid changes during acute cold stress and the slower adaptive changes during long-term cold acclimation.

Upon acute cold stress there is an unmasking of mitochondrial proton conductance pathways. This unmasking is characterized by an increase in purine nucleotide binding, independent of protein synthesis and probably mediated by NE and associated with structural changes in the mitochondria. It most probably contributes to the increased oxygen consumption and heat production of adipocytes upon NE administration. Several questions arise concerning the mitochondrial changes which occur in this early phase of cold exposure.

What is the relation of these changes to the mechanism of the calorigenic action of NE on BAT?

Current hypotheses propose that NE increases the cytoplasmic concentration of "antinucleotides" which displace bound purine nucleotides from mitochondrial proton conductance sites and induce an enhanced energy dissipation. "Antinucleotides" which have been proposed are fatty acids, fatty acyl CoA and alkaline pH (see literature review p. 47-49). There

is still uncertainty about the identity of the physiological mediator of NE action. The most promising tool for solving this problem is the method recently developed by Nicholls (139) to measure directly the mitochondrial proton electrochemical gradient in intact cells. Mitochondrial energy state in intact cells has never been determined. The mechanism of action of NE could probably be deduced from the induction of energy dissipation by some of the possible mediators. Unmasking of purine nucleotide sites is not incompatible with the "antinucleotide" hypothesis. Both phenomena are not mutually exclusive events. On the contrary, they may very likely support and enhance each other. It is even possible that unmasking of proton conductance pathways could be sufficient by itself to induce mitochondrial energy dissipation in vivo by rendering the number of sites greater than the amount of nucleotides available in the cell for binding.

What is the mechanism of the NE-induced unmasking of purine nucleotide binding sites in BAT mitochondria?

It is possible that a reorganization of mitochondrial inner membranes is responsible for the unmasking of proton conductance pathways. Upon cold stress, mitochondrial internal structure changes from a "dotted" appearance to a parallel arrangement of cristae increasing the inner membrane surface. An increase in purine nucleotide binding and a similar transformation of mitochondrial structure are obtained in vitro by incubating isolated BAT mitochondria at alkaline pH in a KCl medium. This in vitro unmasking excludes the involvement of cAMP or cGMP-dependent phosphorylation or the action of cytoplasmic proteases or lipases.

The possibility that the calorigenic effect of NE on brown adipocytes

could be mediated via an intracellular alkalinization as proposed by Chinet and co-workers (42) is attractive since a high concentration of OH^- could serve both as an uncoupling agent and as an "antinucleotide". If so, it should be possible to demonstrate the induction by NE of a change in intracellular pH in vitro and in vivo and that this change is responsible for increasing mitochondrial energy dissipation and adipocyte respiratory rates. Acute cold exposure should also induce a change in intracellular pH. Techniques for measuring intracellular pH in vitro and in vivo are presently available (30). This information is absolutely necessary in order to accept the hypothesis of an intracellular increase in pH as being the physiological messenger of NE to the mitochondria.

It is however very difficult to decide whether the changes in mitochondrial internal structure and osmotic properties are the cause or the effect of unmasking. Further research is obviously needed at the level of the mitochondria during unmasking to answer this question. For example, what are the ion contents of BAT mitochondria after cold exposure or NE infusion, particularly K^+ and PO_4^{--} which are the main mitochondrial osmotic supports. Also of interest are the levels of Mg^{++} and Ca^{++} which are known to vary in BAT mitochondria upon cold acclimation (56) and in heart mitochondria after a subcutaneous injection of isoproterenol (193). It is also known that Mg^{++} is lost to a large extent from liver mitochondria incubated for 20 minutes at 37°C in KCl-sucrose medium (148). The question is whether the increase in purine nucleotide binding depends on the increase in inner membrane surface exposing sites that were present but unavailable for binding or depends on modification of a binding protein or of its environment.

Which adrenergic receptors control the induction of mitochondrial respiration?

BAT possesses both α and β-adrenergic receptors. It is not known which receptor type controls the unmasking of proton conductance pathways and subsequent energy dissipation. It would be possible to investigate this problem by trying to prevent the NE-induced increase in purine nucleotide binding with α or β-antagonist. Similar conclusions could probably be reached by studying the unmasking effects of α or β-agonists administered by intravenous infusion.

Upon long term cold exposure, there is an hypertrophy of BAT and a proliferation of mitochondria within the tissue. The mitochondrial cristae become more numerous and arranged in a parallel fashion. Simultaneously, there is an increase in the concentration of proton conductance pathways of the mitochondria. All these changes regress rapidly and at the same rate when the C-A rats are returned to a warm environment. Cytosolic protein synthesis is required for the change in mitochondrial composition which leads to the increase in proton conductance pathways. Although NE may be, at least in part, responsible for the BAT hypertrophy, the changes in mitochondrial composition are not mediated by NE. The increased concentration of proton conductance pathways in C-A rats does not alter the total in vitro respiratory capacity of mitochondria and a regulatory function must be proposed. The increased concentration of proton conductance pathways in C-A rats most probably contributes to the enhanced capacity of the animals for NST. Several questions arise concerning the changes taking place during this adaptive phase of cold acclimation.

What is normally the rate limiting step for BAT mitochondrial respiration?

From the results obtained in this thesis and comparison with literature data, it is concluded that the respiratory capacity of brown fat mitochondria is limited by the rate of substrate oxidation rather than rate of proton re-entry, at least in vitro, with the incubation conditions used. More conclusive evidence could be obtained by measuring rate of proton re-entry and substrate oxidation on the same mitochondrial preparation respiring with a large number of different substrates.

It is suggested that the increased concentration of proton conductance pathways results in the presence of a larger number of sites than can be blocked by the nucleotides present in the cytoplasm. Thermogenesis would then become dependent on substrate availability, itself under β-adrenergic control.

How are the rates of protein synthesis and degradation altered in BAT during cold acclimation and deacclimation?

There is evidence that selective synthesis of 32 000 M.W. polypeptide starts within 24 hrs of cold exposure (Dittmar E. and Himms-Hagen J., unpublished data). A selective decrease in rate of degradation also appears likely. No information is available concerning mitochondrial or tissue protein turnover during deacclimation. Such findings would help to clarify the mechanism by which changes occur in mitochondrial protein composition and the possible relation between mitochondrial components such as the one possibly existing between the 32 000 M.W. and 96 000 M.W. polypeptides. These studies would however involve the difficult measurement of protein turnover rate in non-steady state condition (223). Upon deacclimation, because of the similarity of the

time-courses of regression of cold-induced tissue or mitochondrial changes, it is more likely that a general tissue degradation is involved. In this regard, measurements of cytoplasmic or lysosomal protease activity may be very informative. It is nevertheless obvious that BAT appears to be a wonderful model for the study of regulation of protein turnover in mammalian cells.

What is (are) the hormone(s) responsible for the adaptive changes in BAT upon cold acclimation?

As mentioned in the literature review (pp. 51-58), no single hormone can be designated as the "causal" agent for the changes in the thermogenic capacity of BAT upon cold acclimation. NE is not responsible for the changes in mitochondrial composition and mimics only imperfectly the effects of cold on BAT itself. It is also not known whether NE is responsible for the maintenance of BAT in C-A rats. It should be possible to investigate further this aspect of NE action from the administration of NE in C-A rats kept at 28°C.

Recent findings by Rothwell and Stock (241) indicated that voluntary overfeeding in rats induces a growth of BAT and an increased calorigenic response to catecholamines in the obese rats. It appears then possible that diet-related hormones could be responsible for brown fat transformations. The three main diet-related hormones are insulin, glucagon and growth hormone. Growth hormone is indeed involved in protein, lipid and carbohydrate metabolism and their clearance from the plasma (3, 97, 165). A preliminary experiment in this laboratory indicated that growth hormone might induce an increase in the concentration of mitochondrial proton conductance pathways in BAT. Injections of growth hormone for 5 days in rats kept at 28°C induced an increase in purine nucleotide binding by BAT

mitochondria. The GDP binding date are: saline-treated 0.068 nmoles/mg protein and growth hormone-treated 0.173 nmoles/mg protein. This experiment needs to be repeated and the calorigenic effect of NE in the treated rats as well as their BAT mitochondrial protein composition and proton conductance need to be studied.

Relation of the research described in this thesis to work in other areas

The results of Stock and Rothwell have a much greater importance than the simple possible identification of regulatory hormones.

Is BAT only involved in response to cold?

The essential part of their findings is the involvement of BAT in overall energy balance. It appears that growth of BAT upon overfeeding occurs to counteract the increased energy intake and that it may actually determine metabolic efficiency and resistance to obesity. High metabolic efficiency is present in various types of genetically obese rodents (285). The positive energy balance that leads to obesity in these animals is due to a combination of hyperphagia and of an elevated metabolic efficiency. In ob/ob mice, increased metabolic efficiency is due to a reduced maintenance requirement. These animals have a thermoregulatory defect, detectable very early in life before obesity is visually apparent (284). This thermoregulatory defect is characterized by a reduced ability to respond to cold environments (53). Adult ob/ob mice die of hypothermia when exposed for few hours at 4°C. They also have a reduced calorigenic response to catecholamines (283). Furthermore, a mitochondrial defect in BAT of the obese mice has been demonstrated (133). They have a reduced binding of purine nucleotides to their mitochondria and the cold-induced unmasking of proton conductance sites occurring in lean animals does not

occur in obese mice. It was then postulated that reduced operation of mitochondrial proton cycling in BAT of obese mice kept at temperature below thermoneutral could result in less wasting of substrates for heat production and thus in a greater metabolic efficiency. These results point to a major role for BAT in obesity. The well-established involvement of BAT in nonshivering thermogenesis must now be viewed within the more general concept of energy balance in living mammalian organisms. There is no doubt that the recent demonstration of occurrence of BAT in adult humans (241, 273, 274) will certainly stimulate intensive research into the exact role and importance of BAT in the control of obesity (121).

In addition, the adaptable nature of BAT, its responsiveness to various hormones, its rate of growth and regression and the amplitude of the changes observed certainly also make it an ideal system for numerous studies most particularly as regard to regulation of lipid metabolism and mitochondrial biogenesis.

APPENDIX A

THEORY AND PRACTICE OF THE COULTER ELECTRONIC PARTICLE COUNTER

The Coulter Counter automatically determines the number and volume of particles suspended in an electrically conductive solution. Fig. a. shows a simplified diagram of the counting assembly. Basically, it consists of a tube with a small circular aperture at its lower end, two immersed platinum electrodes at opposite sides of the orifice and a mercury manometer as a metering device. The operation of the Coulter counter depends on the difference in electrical resistance between the electrolyte and the particles suspended in it. When a potential difference is set up between the two platinum electrodes, the resistance is a function of the conductivity of the medium and the aperture of the tube. As a high resistivity particle passes through the orifice where the current is restricted to a small sensing zone, it displaces its own volume of electrolyte medium and causes a momentary increase in resistance. Since volume is independent of shape, it does not matter whether particles being measured by the Coulter Counter are rods, spheres, or spirals. The resistance changes induced by the particles going through the aperture are amplified and counted as voltage pulses which are proportional to the volume of the particles. By means of a pulse height analyzer (Channelyzer), it is possible to determine the volume distribution of the particles as well as their number. Very low detection limits can be obtained with the use of small apertures. For example, a 30 μ aperture allows the sizing and counting of particles with diameters as small as 0.45- 0.5 μm while a 19 μ aperture can extend the detection limit to

0.35-0.4 μm particle diameter. The 10 μ aperture used in this study has a low detection limit of 0.2 μm.

The Coulter Counter has previously been designed to count and size blood cells, bacteria, spermatozoa and other particles of biological origins. It has also been successfully used to size and count mitochondria (90, 91, 96). It appears that the membranes of the mitochondria or other biological particles are responsible for the high electrical resistance that allows their recording by the counter (91, 210). However, only overall mitochondrial volume can be determined by the Coulter Counter and not the conformational state of the inner membrane (246). The mitochondrial volume experimentally determined with the Coulter Counter agrees well with the volume measured from electron micrographs of isolated liver or heart mitochondria (90). It has also been found that the errors in the use of the counter for the determination of the number of mitochondria were very small when compared with other counting technique such as light microscopy (96). The mitochondria are stable in the diluent solution (90, 91). No disintegration of the mitochondria was reported and only a small swelling (about 8%) occurred over one hour. Contaminating subcellular organelles were shown to have little influence on the experimentally determined size distribution (90). However, counting errors may be introduced due to aggregation of the mitochondria and coincidence. Those problems do not appear to be significant when large dilutions of the mitochondrial suspension are employed (91). Coincidence occurs when two or more particles enter simultaneously the effective counting volume of the apparatus. They are then recorded as a single particle and counts are lost. The need for coincidence correction only applies when large apertures which have a much larger effective counting volume are used or

when the mitochondrial concentration is too high. The Coulter Counter technique is then very useful and reliable for the determination of the number and size of mitochondria when using small aperture such as the 10 μ one used in this study and large dilution of the mitochondrial suspension.

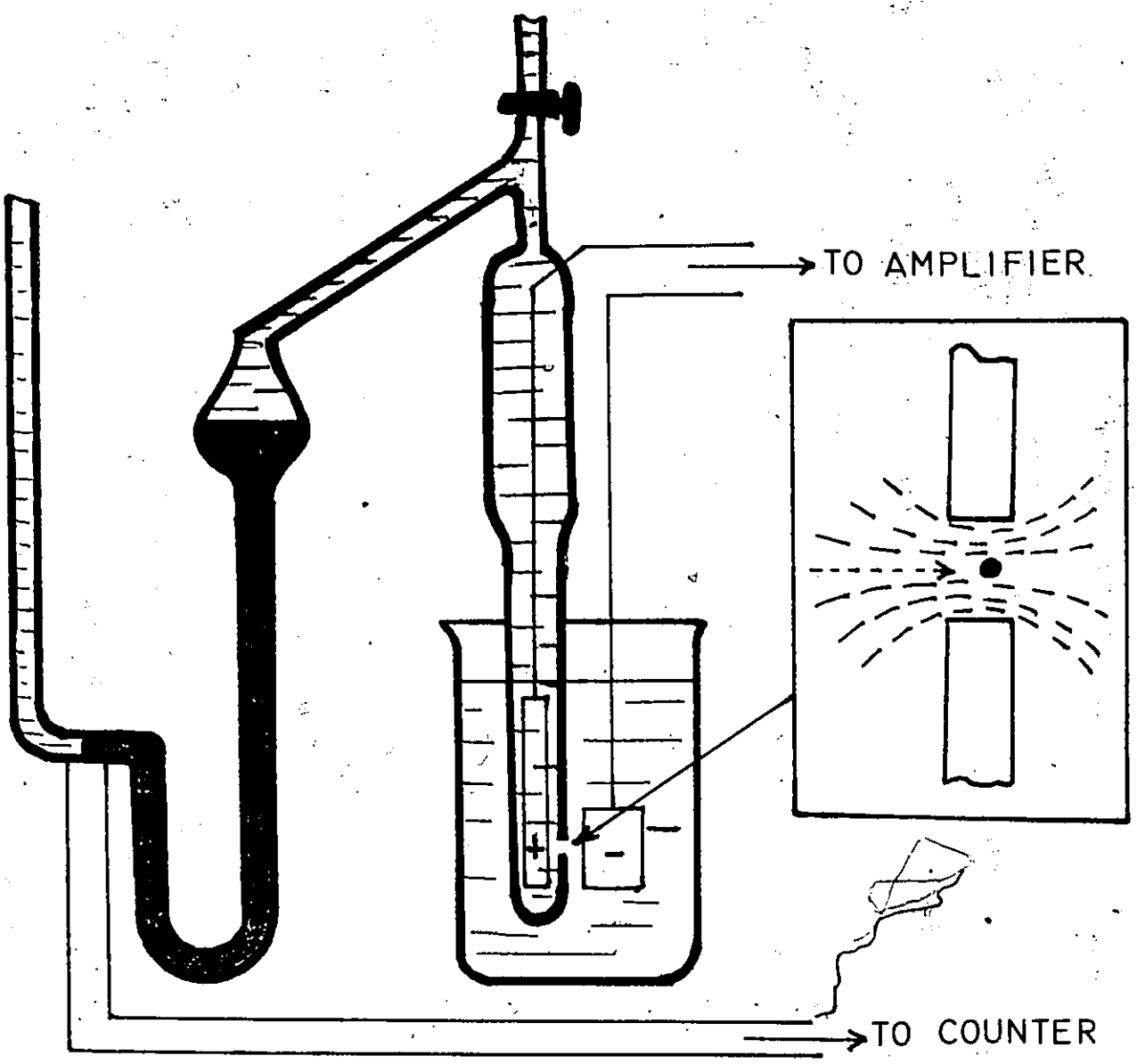


FIG. a. Counting assembly of the electronic particle counter.

As each particle passes through the orifice, it displaces its own volume of diluent within the orifice. The electric current paths are then distorded or modulated by the presence of this high resistivity particle. A momentary change in the resistance value between the electrodes is then recorded, amplified and analysed by the channelyzer.

APPENDIX B

PROCEDURE FOR DEHYDRATION OF SAMPLES AND EMBEDDING IN VESTOPAL W

- Mitochondrial pellet fragments are in 3.0 ml osmium tetroxide solution

- 50% ethanol
  add equal volume, remove half
  wash 2 x more
  wait 3 min.
  wash 1 x
  wait 5 min.

- 75% ethanol
  add equal volume, remove half
  wash 2 x more
  wait 3 min.
  wash 1 x
  wait 5 min.

- 95% ethanol
  add equal volume, remove half
  wash 2 x more
  wait 3 min.
  wash 1 x
  wait 5 min.

- 100% ethanol
  add equal volume, remove half
  wash 4 x more
  wait 15 min.
  wash 10 x more, rapidly

- styrene
  add equal volume, remove half
  wash 4 x more
  wait 5 min.
  wash 5 x more
  wait 5 min.

- styrene + fresh
  Vestopal (initiator + activator) (1:1 mixture)
  remove as much styrene as possible
  add styrene-Vestopal mixture
  on rotator of 10 min.

- Vestopal
  remove mixture
  add Vestopal
  on rotator for 10 min.
  change solution and transfer tissues to clean vials
  on rotator for 10 min.
  change solution
  on rotator for 2 days

- Transfer samples to capsules almost filled with Vestopal (with toothpicks)
- Transfer to oven at 60°C and leave for 4 days.

APPENDIX C

THEORY AND PRACTICE OF THE DETERMINATION OF MITOCHONDRIAL PROTON ELECTROCHEMICAL GRADIENT

The proton electrochemical gradient (Δp) across the mitochondrial inner membrane is defined by the following relationship: $\Delta p = \Delta E - 59\ \Delta pH$ where ΔE is the membrane potential and ΔpH the transmembrane pH gradient.

The membrane potential (ΔE) is measured from the distribution of $^{86}Rb^+$ inside and outside of the mitochondria in presence of valinomycin, a K^+ ionophore. In excess of valinomycin, K^+ equilibrates quickly across the inner membrane in response to the existing membrane potential (244). $^{86}Rb^+$ is equally well transported by valinomycin as K^+ and it is assumed that the distribution of $^{86}Rb^+$ equals the distribution of K^+ so that the membrane potential can be calculated from the relation:

$\Delta E = 59 \log_{10} (Rb^+)_i/(Rb^+)_o$, where $(Rb^+)_i$ and $(Rb^+)_o$ represents the concentrations of cation in the internal (matrix) and external (medium) phases, respectively (242, 244). The transmembrane ΔpH gradient is defined by the distribution of weak acid (acetate) or base (methylammonium) inside and outside of the mitochondria. The ability of these compounds to monitor ΔpH is based on the assumption that the membrane is impermeable to charged species (A^- or $MeNH_4^+$) and that the concentration of uncharged forms (CH_3COOH or $MeNH_3$) are equal on both sides of the membrane. The distribution is governed by the Henderson-Hasselbach equation and

$\Delta pH = \log_{10} (A)_o/(A)_i = \log_{10}(B)_i/(B)_o$

Thus, if $\Delta pH < 0$ (i.e. if the matrix is more alkaline)

$\Delta p = 59 \log_{10} (Rb)_i/(Rb)_o + 59 \log_{10} (Ac)_i/(Ac)_o$

In this case, methylammonium (base) is excluded from the matrix and can be used to determine the extra-matrix volume (volume trapped in pellet). If $\Delta pH > 0$, (i.e. the matrix is more acidic),

$\Delta p = 59 \log_{10} (Rb)_i/(Rb)_o - 59 \log_{10} (MeNH_4^+)_i/(MeNH_4^+)_o$.

In the latter case, acetate is excluded from the matrix and can be used for the estimation of the extra-matrix volume.

The method thus consists of incubating mitochondria in a suitable medium supplemented with ^{14}C-methylammonium, ^{3}H-acetate and ^{86}RbCl in presence of valinomycin. Mitochondria are separated from the incubation medium by centrifugation and the distribution of radioactivity between the pellet and the incubation medium is measured. Matrix volume is determined as the ^{14}C-sucrose impermeable space by a gravimetric method on parallel samples.

The suitability of the centrifugation procedure to measure ion distribution has been questioned on the basis that anaerobiosis is reached rapidly in the pellet with subsequent alteration of the ion distribution between inner and outer spaces. However, it has been shown that similar results are obtained whether or not separation of mitochondria from medium is carried out. Also, although a shift from the matrix to the extra-matrix space certainly occurs within the pellet after centrifugation, this does not affect the total ion content of the pellet. The ion distribution between the matrix space and the medium during incubation is reflected in the ion distribution between total pellet (matrix + extra-matrix space) and supernatant, and may be modified only to the extent that there is ion diffusion from the pellet to the supernatant. This is however too slow, due to the restricted surface, to affect significantly the ion content in the pellet (5).

The calculations of Δp were carried exactly as described by Nicholls (197). The counts present in the pellet for each isotope were converted into their "volume or space" equivalent as though the isotopes had retained the same concentration (cpm/ml) in the pellet as in the incubation medium.

"space" $= \frac{V}{(N/n)-1}$ µl, where V is the volume of the incubation medium

N is the total count of the isotope in the medium

n is the counts present in the pellet.

If these "spaces" are "r, c, h" for rubidium, methylammonium and acetate respectively, and the matrix volume, determined as the sucrose space, is "m", the degree of concentration of each isotope within the matrix is given by the following relationships:

$(Rb)_i/(Rb)_o = r - v/m$

$(MeAm)_i/(MeAm)_o = c - v/m$

$(Ac)_i/(Ac)_o = h - v/m$ where v is the extra-matrix volume of incubation entrapped in the pellet.

When $\Delta pH < 0$, $v = c$; when $\Delta pH > 0$, $v = h$.

So, when $\Delta pH < 0$, ΔpH is calculated from the relation

$$\Delta pH = \log_{10} (Ac)_i/(Ac)_o = \log_{10}\left(\frac{h-c}{m}\right).$$

When $\Delta pH > 0$, $\Delta pH = \log_{10} (MeAm)_i/(MeAm)_o = \log_{10}\left(\frac{c-h}{m}\right)$.

The dependency of $(c-h)/m$ upon ΔpH is illustrated in Fig. B. An example of the calculations is given on the next page.

The calculation of the results are exactly as described by Nicholls.

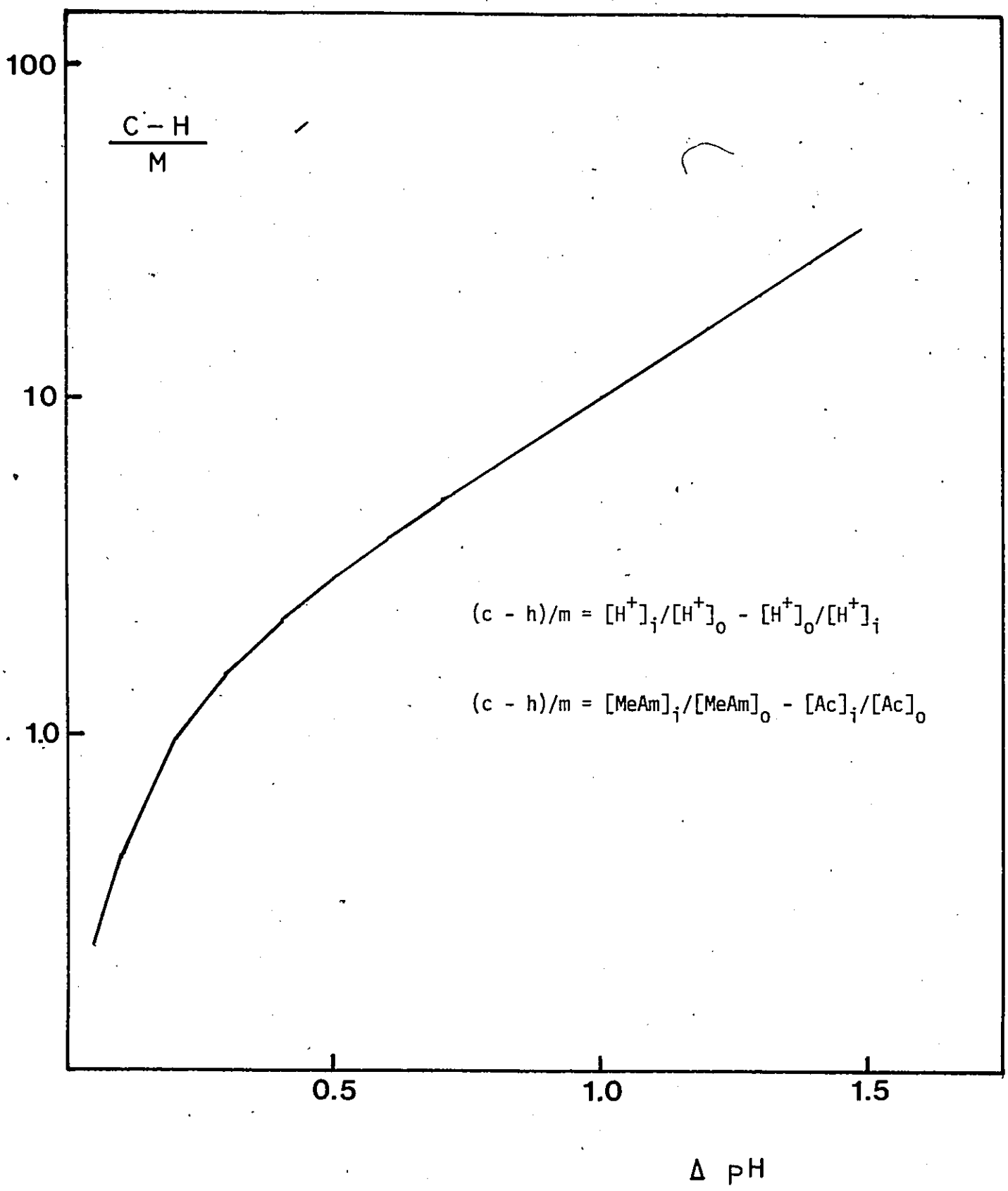
Example: from experiment #6, cold acclimated rat,

a) basic medium, no addition

b) basic medium, 1mM GDP, 0.5% albumin

Radioactivity in tubes	^{3}H-Acetate	1 036 950 cpm/ml	Total volume= 1.0 ml	
	^{14}C-Methyl-ammonium	151 050 cpm/ml	Matrix volume= 0.64 µl/ mg prot.	
	^{86}RbCl	35 080 cpm/ml	Mito. Protein = 0.795 mg/ml	
		a)	b)	
cpm in pellets	^{3}H-	2 021	11 953	cpm are corrected for background and crossovers. Crossovers: 40% ^{86}Rb cpm in ^{14}C-channel 14% ^{14}C cpm in ^{3}H-channel
	^{14}C-	917	869	
	^{86}Rb-	161	3 495	
Apparent "spaces (µl)	^{3}H- (h)	1.953	11.66	From $\frac{V}{(N/n) - 1.0}$ V: Total counts of isotope in tubes n: Counts retained in pellet v: Total volume (µl)
	^{14}C- (c)	6.11	5.78	
	^{86}Rb- (r)	4.62	100.67	
Matrix conc.	Ac_i/Ac_o	-	11.61	= (h-v)/m; = (c-v)/m; = (r-v)/m; v: extra-matrix volume (µl); m: matrix volume (µl); If ΔpH > 0, v= h; If ΔpH < 0, v= c
	$MeAm_i/MeAm_o$	8.21	-	
	Rb_i/Rb_o	5.27	207.12	
-59 ΔpH		-54.58	+ 63.13	ΔpH from graph, next page. From 59 log (Rb_i/Rb_o)
ΔE		+42.61	+136.66	
Δp		-11.97	+199.79	From Δp = ΔE - 59 ΔpH

FIG. b.



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