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ADAPTIVE ALTERATIONS IN SARCOLEMMA OF SKELETAL MUSCLE IN COLD-ACCLIMATED RATS

BY JACQUES CERF

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.

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF MEDICINE
UNIVERSITY OF OTTAWA

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A ASSIA ET ANDRE

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I would also like to thank Dr. M. Kates for the use of his laboratory facilities and Ms Elizabeth Dittmar for her skillful technical assistance. Finally, and in a very special manner, I thank Denise for her constant encouragement throughout this work.

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ABBREVIATIONS

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
CA	cold-acclimated
cyclic AMP	adenosine 3',5'-monophosphate
(±)[^{3}H]DHA	(-)[^{3}H]dihydroalprenolol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(aminoethylether)N,N'-tetraacetic acid.
GppNHp	5'-guanylyl imidodiphosphate
NA	noradrenaline
NADH	β-nicotinamide adenine dinucleotide, reduced form.
NST	nonshivering thermogenesis
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PS	phosphatidylserine
Sph	sphingomyelin
Tris	trishydroxymethylaminomethane
WA	warm-acclimated

SUMMARY

A cold-acclimated rat shows a characteristic absence of shivering when it is placed in a cold environment. To maintain its body temperature constant, the cold-acclimated rat relies on nonshivering thermogenesis, which is known to be a calorigenic response to noradrenaline secreted by sympathetic nerve endings. The cold-acclimated rat differs from the control (warm-acclimated) rat in that it shows an increased calorigenic response to catecholamine administration.

The principal sites of nonshivering thermogenesis are skeletal muscle and brown adipose tissue. Much work has been done on skeletal muscle and brown adipose tissue mitochondria to provide an explanation for the biochemical mechanism of nonshivering thermogenesis. The research reported in this thesis concerns skeletal muscle plasma membrane. A possible mechanism of nonshivering thermogenesis in muscle might involve changes in the enzymes present in the plasma membrane which have been shown to respond to the presence of catecholamines, namely adenylate cyclase and Na,K-ATPase. An increase in adenylate cyclase and/or Na,K-ATPase activity or in their responsiveness to catecholamines in the cold-acclimated rat might underlie the enhanced calorigenic response to catecholamines.

Two different techniques were used to isolate skeletal muscle plasma membranes (sarcolemma). In technique

I, strong salt concentrations allowed extraction of the contents of tubes of sarcolemma. In technique II, plasma membranes were isolated as small vesicles by sucrose density-gradient centrifugation. The morphology of preparation I was studied by phase contrast microscopy. Contamination by mitochondria was assessed by measurement of cytochrome oxidase. The following characteristics of sarcolemma were studied:

- 5'-nucleotidase activity.
- Na,K-ATPase activity.
- adenylate cyclase activity.
- number of β-adrenergic receptor sites.
- lipid composition.
- polypeptide composition.

The results are summarized in two steps, the first concerning membrane characterization, the second, the effect of cold acclimation on properties of the sarcolemma.

The major findings concerning membrane characterization are the following:

- similar minor contamination by mitochondria in both preparations.
- higher 5'-nucleotidase activity in preparation I.
- higher Na,K-ATPase activity in preparation II, but no inhibition by ouabain in this preparation.
- much higher Mg-ATPase activity in preparation II.
- higher adenylate cyclase activities (basal , NaF-, GppNHp-, noradrenaline- and GppNHp + noradrenaline-

stimulated) in preparation II.

- similar number of β-adrenergic receptor sites (assessed by (-)[^{3}H]dihydroalprenolol binding) in both preparations.
- similar phospholipid composition in both preparations.
- similar degree of unsaturation of each phospholipid fraction in both preparations.
- differences in the fatty acid composition in the PS + PI and sphingomyelin fractions.
- similar cholesterol content in both preparations.
- similar polypeptide patterns in the low molecular weight range (< 90 000), different in the high molecular weight range (> 90 000).

It is concluded that both techniques isolate sarcolemma. However, the material isolated differs, both morphologically and in its metabolic properties, in the two preparations. The higher Na,K-ATPase, Mg-ATPase, adenylate cyclase activities, the lower 5'-nucleotidase activity, and the resistance of the Na,K-ATPase to inhibition by ouabain seen in preparation II suggests that the vesicles isolated by technique II may be inside-out. β-Adrenergic receptors are present in both preparations.

As far as cold acclimation is concerned, similar results are obtained with preparation I and II from cold-acclimated rats. The major findings are the following:

- no change in the yield of plasma membrane protein.
- similar contamination by mitochondria.

- similar 5'-nucleotidase activity.
- increase in the activity of Na,K-ATPase but no effect of noradrenaline on the enzyme.
- no consistent increase in Mg-ATPase activity.
- no change in adenylate cyclase activities (basal, NaF-, GppNHp-, noradrenaline-, GppNHp + noradrenaline-stimulated).
- decrease in the number of β-adrenergic receptors with resensitization of the receptors by GppNHp.
- no change in the lipid content but increased degree of unsaturation of fatty acids in the PS + PI fraction.
- no change in the polypeptide composition.

It is concluded that the sarcolemma of skeletal muscle is altered in the cold-acclimated rat. The increase in Na,K-ATPase activity may be due to an altered lipid environment (increased unsaturation in the PS + PI fraction) since no change in polypeptide composition was detectable. The decrease in concentration of β-adrenergic receptors may be due to a desensitization by high circulating levels of catecholamines since resensitization with guanylyl imidodiphosphate can be achieved in vitro. These modifications do not provide an explanation for the enhanced calorigenic response to catecholamines in the cold-acclimated rat. It remains a possibility that the regulation in vivo by catecholamines of the plasma membrane components studied is not detectable in vitro.

CHAPTER I: INTRODUCTION AND STATEMENT OF THE PROBLEM

The ways in which mammals react to living in a cold environment can be classified into three types:

(1) The animal hibernates

(2) The animal undergoes a metabolic adaptation which allows it to maintain its body temperature by increased heat production in the absence of shivering (nonshivering thermogenesis)

(3) The animal neither undergoes a metabolic adaptation to allow increased heat production nor hibernates. Shivering persists as the main heat producing mechanism in such animals, although adaptive circulatory changes may occur which permit reduced heat loss and thus, reduce the intensity of shivering.

Examples of mammals which react in these ways are (1) hamster, (2) rat, (3) man. This thesis concerns the metabolic adaptation which occurs in the rat living in a cold environment and allows increased heat production by nonshivering thermogenesis.

When a rat is first exposed to cold, shivering thermogenesis is the main mechanism used to maintain a constant body temperature. However, shivering gradually decreases with time and after four weeks, the rat uses nonshivering thermogenesis to maintain a constant body temperature. At this time, the rat is said to be cold-acclimated.

When the work described in this thesis was started, it was generally believed that skeletal muscle was the major

site of nonshivering thermogenesis. More recently, brown adipose tissue has been demonstrated to be a major site, with some nonshivering thermogenesis occuring in skeletal muscle. Nonshivering thermogenesis is known to be controlled by the sympathetic nervous system and, the cold-acclimated rat, in which the capacity for nonshivering thermogenesis is increased, is characterized by a four-fold increase in metabolic rate when infused with noradrenaline compared with the two-fold increase seen in the non-acclimated rat. This enhanced response to catecholamines is what differentiates biochemically the cold-acclimated rat from the control (or warm-acclimated) rat when both are in a warm environment.

There are three cellular locations where an adaptive change might occur to bring about a change in response to catecholamine action:

- The plasma membrane
- The mitochondrion
- The cytoplasm

The work described in this thesis was done to find out if there were any changes in the plasma membrane of skeletal muscle of the cold-acclimated rat which might account for all or part of the increased capacity of this tissue to respond to noradrenaline by an increase in metabolic rate.

Initial experiments showed that no isolation technique reported in the literature at that time was completely satisfactory. Therefore, modified methods were developed,

one involving lithium bromide extraction, the other a milder technique. In order to establish the nature of the material isolated, various marker enzymes and proteins characteristic of plasma membranes were measured (Na,K-ATPase, adenylate cyclase, beta-adrenergic receptors, 5'-nucleotidase) as well as a marker enzyme for mitochondria (cytochrome oxidase). In addition, the lipid and protein composition and the morphology of the preparations were assessed. In all experiments, material from cold-acclimated rats was compared with material from warm-acclimated rats. In order to detect a possible difference in action of catecholamines on the sarcolemma of the cold-acclimated rat, the effect of these substances on Na,K-ATPase and on adenylate cyclase was studied as well as the number of beta-adrenergic receptors.

The literature review (chapter II) is divided into several sections corresponding to the different subject areas touched upon in this study. A summary of present knowledge of cold-acclimation is followed by a more detailed discussion of possible mechanisms of nonshivering thermogenesis. Characteristics of plasma membranes in general are then reviewed; this section includes a discussion of the Na,K-ATPase and of its postulated role in thermogenesis, as well as a review of adenylate cyclase and beta-adrenergic receptors. Finally, the literature on the isolation and characterization of sarcolemma is reviewed.

CHAPTER II: LITERATURE REVIEW

PART 1 : COLD-ACCLIMATION AND NONSHIVERING THERMOGENESIS.

The rat is an homeotherm and, as all homeotherms, it is faced with the problem of maintaining its body temperature when it is placed in a cold environment. Two mechanisms are involved in the maintenance of a constant temperature:

- Decrease of heat loss through piloerection and peripheral vasoconstriction.
- Increase in heat production.

This review will deal only with the second mechanism and will show how the increase in heat production occurs mainly through shivering thermogenesis when the rat is exposed to cold and through nonshivering thermogenesis when the rat remains in the cold for a longer period of time. Nonshivering thermogenesis will be defined and reviewed in some detail, as well as the role of the sympathetic nervous system in nonshivering thermogenesis and the sites of this process.

A. Nature of acclimation to cold.

When placed at 6°C, a rat starts to shiver immediately, as measured by the increase in muscle electrical activity (Fig. 1) (78). Shivering thermogenesis is the main mechanism by which the cold-exposed rat maintains a constant body

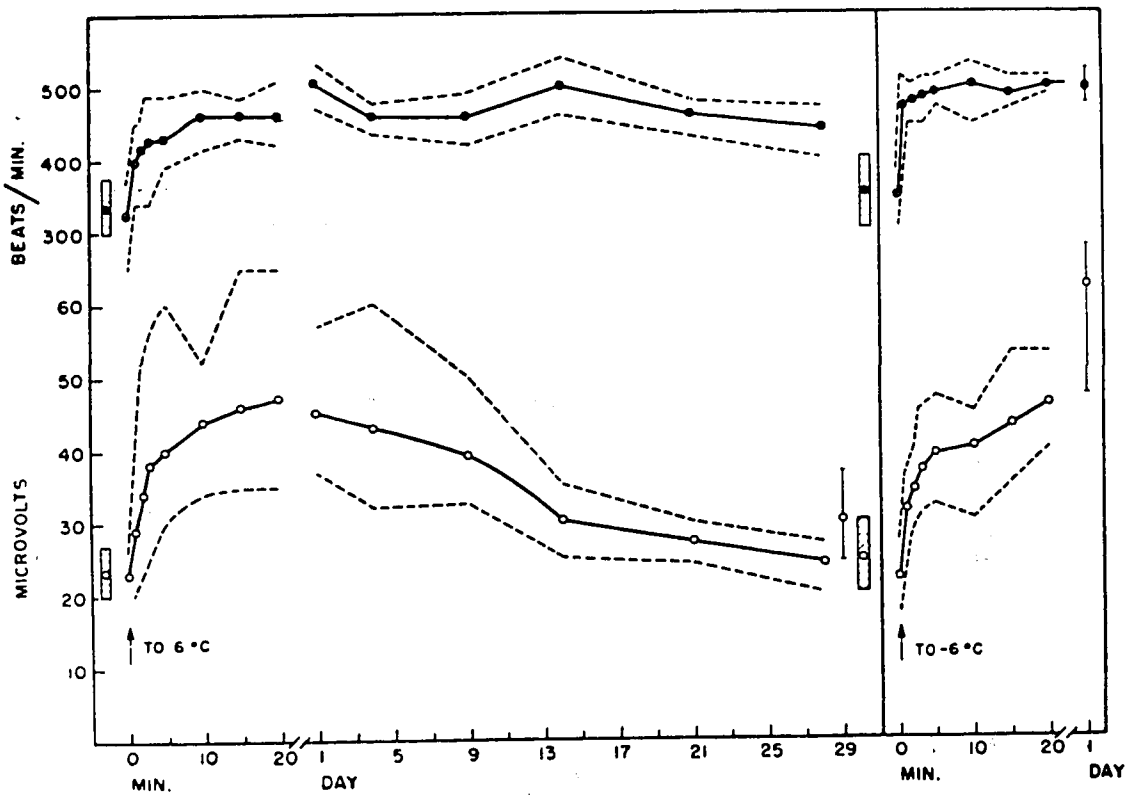

Fig. 1. Increase and decrease in shivering as measured by muscle electrical activity, during acclimation to cold.

Muscle electrical activity as related to time of exposure to 6°C is shown (solid line) in the left-hand part of the diagram (lower tracing). Broken lines show range of variation. Vertical bars at 0 and 29 days show muscle electrical activity in warm-acclimated rats living at 30°C. Upper tracing shows heart rate. At 30 days cold-acclimated rats were moved to -6°C (right-hand part of the diagram). [From Hart et al., (78)].

temperature. If shivering is prevented by tubocurarine (Fig. 2) (45), the rat dies in hypothermia. If the rat is maintained in a cold environment (6°C), shivering gradually decreases and eventually disappears after four weeks (Fig. 1) (78). The rat is said to be cold-acclimated. The cold-acclimated rat is able to keep its body temperature constant when exposed to 6°C, even when treated with tubocurarine, which prevents shivering (Fig. 2) (45). A mechanism other than shivering thermogenesis is responsible for heat production, and is referred to as nonshivering thermogenesis (NST).

B. Definition of nonshivering thermogenesis.

According to Jansky (114), "nonshivering thermogenesis (NST) is a heat-production mechanism liberating chemical energy due to processes which do not involve muscular contractions". This definition includes obligatory NST and regulatory NST. Obligatory (or basal) NST refers to the heat production under the conditions of basal metabolism. Regulatory NST refers to the heat production in animals exposed to temperatures below the thermoneutral zone, and necessary for the maintenance of a constant body temperature. Throughout this thesis, the term NST will refer to regulatory NST.

NST is an adaptive and facultative process:

- adaptive because certain species (e.g. rat, rabbit, guinea pig) have to live in the cold (i.e. become cold-acclimated) for a relatively long time to be able to produce heat

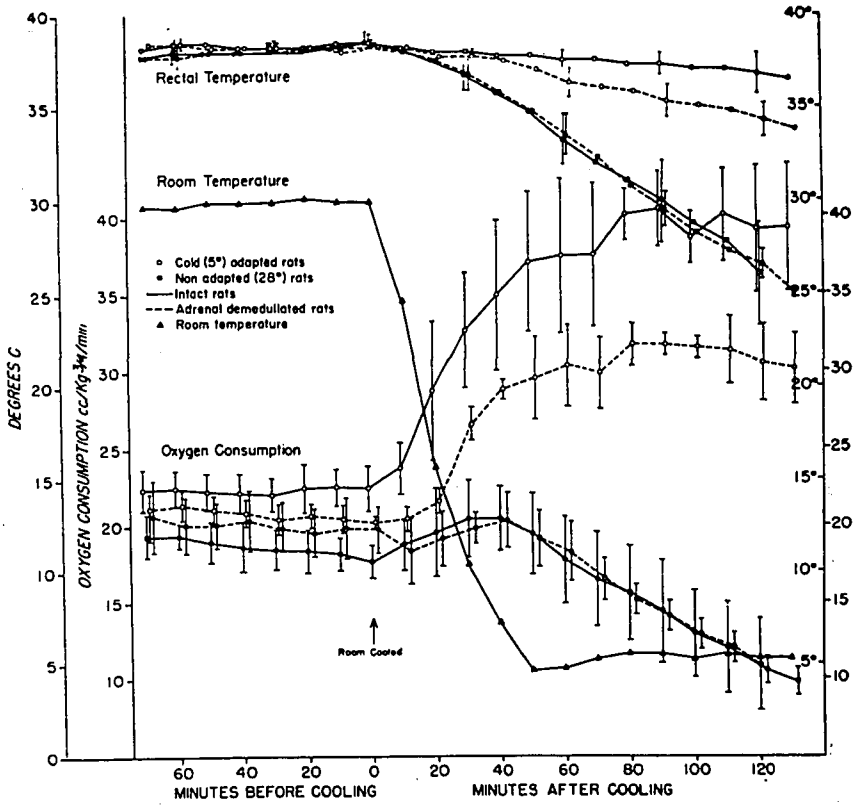

Fig. 2. Increased capacity for nonshivering thermogenesis in cold-acclimated rats.

Metabolic response of curarized, warm- and cold-acclimated rats to lowered ambient temperature is shown (solid lines). Mean oxygen consumption and rectal temperature of each group along with ambient temperature are plotted on ordinate, minutes before and after cooling on abscissa. Standard deviations are indicated by vertical lines. [From Cottle and Carlson (45)].

by NST.

- facultative because NST can be switched on and off according to the animal's needs: on when the animal is placed in the cold and off when it is transferred back to a warm environment. NST can also occur in hibernators (e.g. hamster, bat, ground squirrel) even when they are not acclimated to cold and is especially important during arousal from hibernation. At that time, a great amount of heat is needed by the hibernator in order to overcome the body temperature difference between the hibernating and active state. NST also plays an important role in newborn mammals including man and, in particular, in species that are born immature and incapable of shivering (e.g. lemming, rat). Hence, the physiological significance of NST is quite obvious: a change from 37^{o} to 30^{o}C for a newborn can be considered as a cold stress, especially in the case of species which are born immature.

C. Role of the sympathetic nervous system in acclimation to cold.

The sympathetic nervous system, including the adrenal medulla, is important in the mechanisms leading to reducing heat loss and increasing heat production (85,87). The role of the sympathetic nervous system can be studied by looking at the effects of the stimulation of its activity, by mimicking stimulation with injection of catecholamines and by inhibiting the sympathetic nervous system with a variety of techniques.

C(i). Activation of the sympathetic nervous system.

The measurement of adrenaline, noradrenaline and some of their metabolites in the urine of animals exposed or acclimated to cold gives an indication of the activation of the sympathetic nervous system.

The rate of noradrenaline excretion increases five times upon exposure to cold (Fig. 3) (135) and remains quite high during the acclimation period. Adrenaline excretion, however, although at a maximum after one week of exposure to cold, returns to the low level observed in warm-acclimated rats, after four weeks. Shum and coworkers have reported increased excretion of the metabolites of catecholamines during the first four weeks of cold exposure (210). Normetanephrine excretion reaches a maximum after two weeks and then decreases. 3-Methoxy, 4-hydroxy-phenylglycol (MHPG) excretion, on the other hand, is maximum after one day and remains high. Metanephrine and 3-methoxy, 4-hydroxymandelic acid (MHMA) also show an increased level of excretion.

In conclusion, the increased urinary excretion of catecholamines and their metabolites imply an increased rate of secretion of adrenaline by the adrenal medulla and of noradrenaline by the sympathetic nerve endings.

C(ii). The mimicking effect of catecholamines.

Several lines of evidence show the participation of catecholamines in NST. When noradrenaline is administered, a calorigenic response occurs. It is much greater in cold-

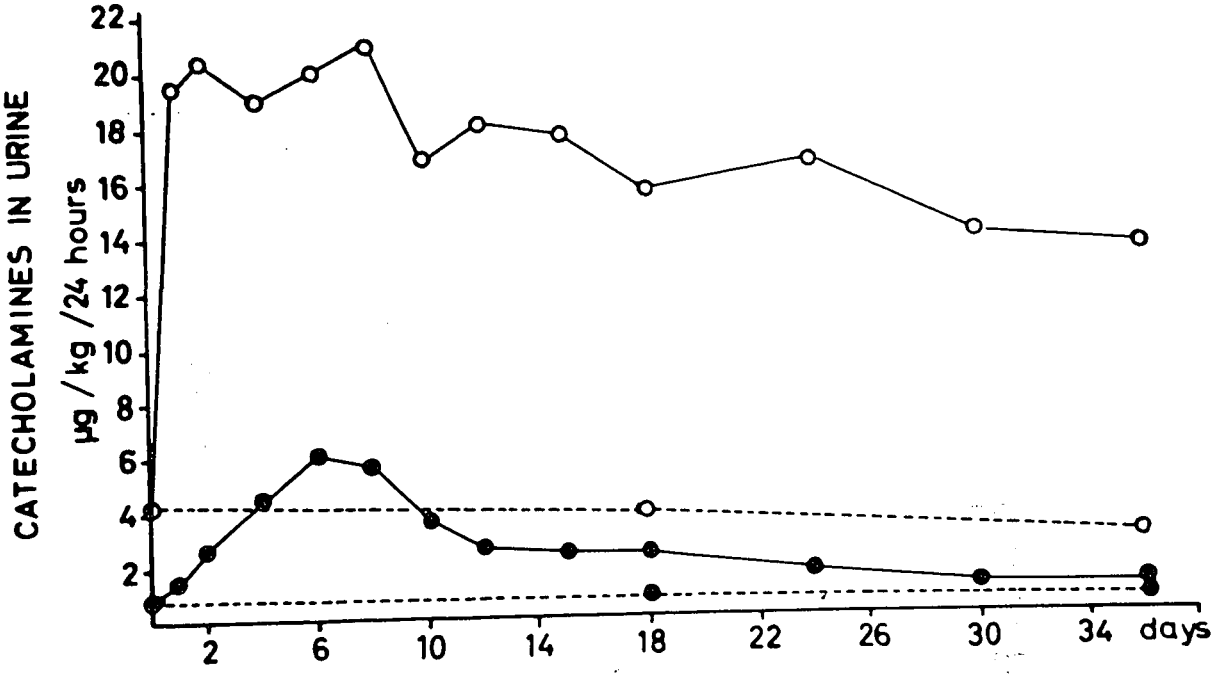

Fig. 3. Urinary excretion of adrenaline and noradrenaline during cold acclimation.

The graph shows the urinary excretion of adrenaline (●) and noradrenaline (○) in rats (170 - 180 g) at 3°C (solid line) and 22°C (dashed line). Each point represents the mean of 6 individual rats. [From Leduc (135)].

acclimated rats than in warm-acclimated rats (104) (Fig. 4) (87). The enhanced calorigenic response in the cold-acclimated rat is also observed in the case of adrenaline administration (Fig. 5) (86). The level of noradrenaline in plasma during infusion of noradrenaline is however lower in the cold-acclimated rat (50); thus, the enhanced calorigenic response to noradrenaline in the cold-acclimated rat is not due to higher circulating levels of noradrenaline as suggested by LeBlanc and Pouliot (134). In addition, the circulating levels of noradrenaline, after its infusion, compare quite well with the concentration needed to stimulate oxygen uptake by tissues in vitro and suggest that these levels approximate the synaptic concentrations of noradrenaline during cold-exposure (49).

The development of the enhanced calorigenic response to noradrenaline during acclimation to cold has been studied by Depocas (49) and is illustrated in figure 6. Comparison of this figure with figure 1 shows that the increase in the enhanced response to noradrenaline coincides with the decrease in shivering.

When a cold-acclimated rat is placed in a warm environment, it gradually loses its ability to produce heat by nonshivering thermogenesis. This process is called deacclimation. The enhanced metabolic response to noradrenaline gradually disappears with deacclimation (14).

A last example will show another type of involvement of noradrenaline in cold-acclimation. LeBlanc and Pouliot treated rats with noradrenaline for a relatively long period

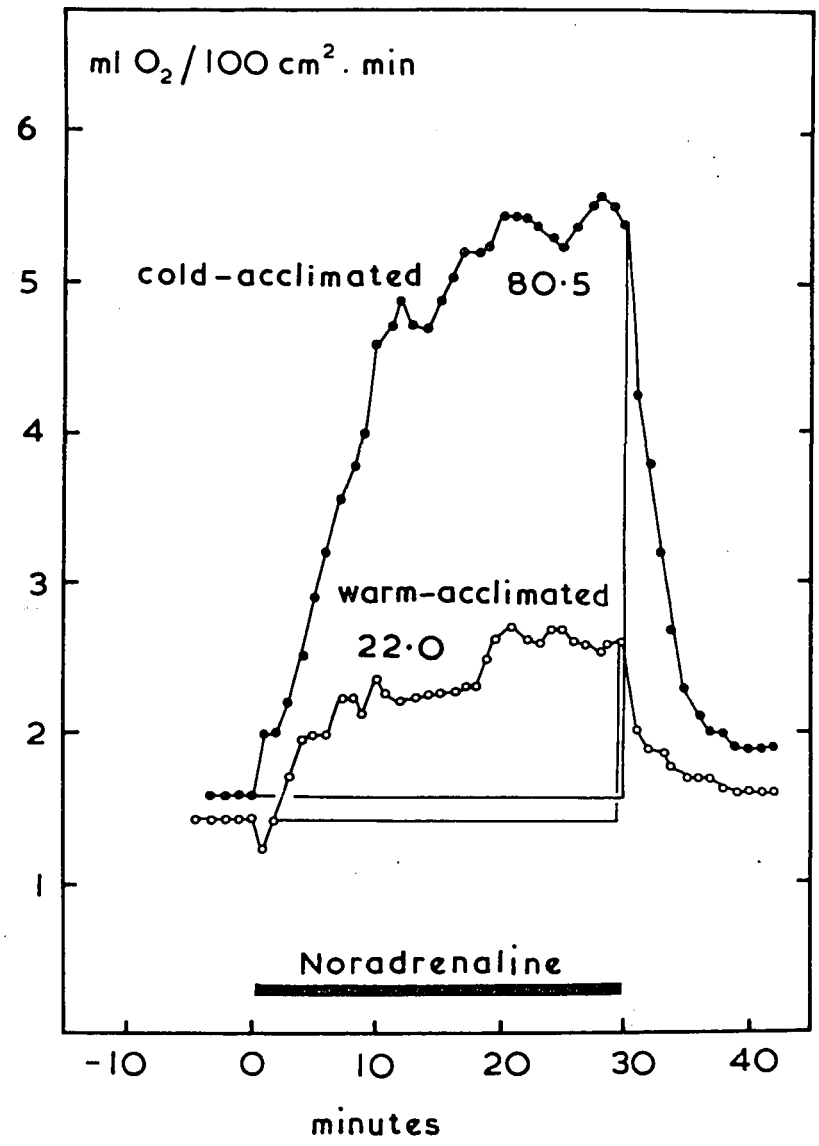

Fig. 4. Enhancement of calorigenic response to noradrenaline by cold acclimation.

Oxygen uptake of a warm- and a cold-acclimated rat during infusion of noradrenaline is shown. Noradrenaline was infused intravenously (0.5 µg/100 cm^2 per min) from 0 to 30 min. Rats were lightly anesthetized with sodium pentobarbital. Warm-acclimated rat weighed 478 g, and cold-acclimated-rat weighed 373 g; they had lived at room temperature (25-28°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 noradrenaline, and they represent total increase in oxygen uptake in ml O_2/100 cm^2/30 min. Increases shown are typical of rats kept under these conditions. [From Himms-Hagen (87)].

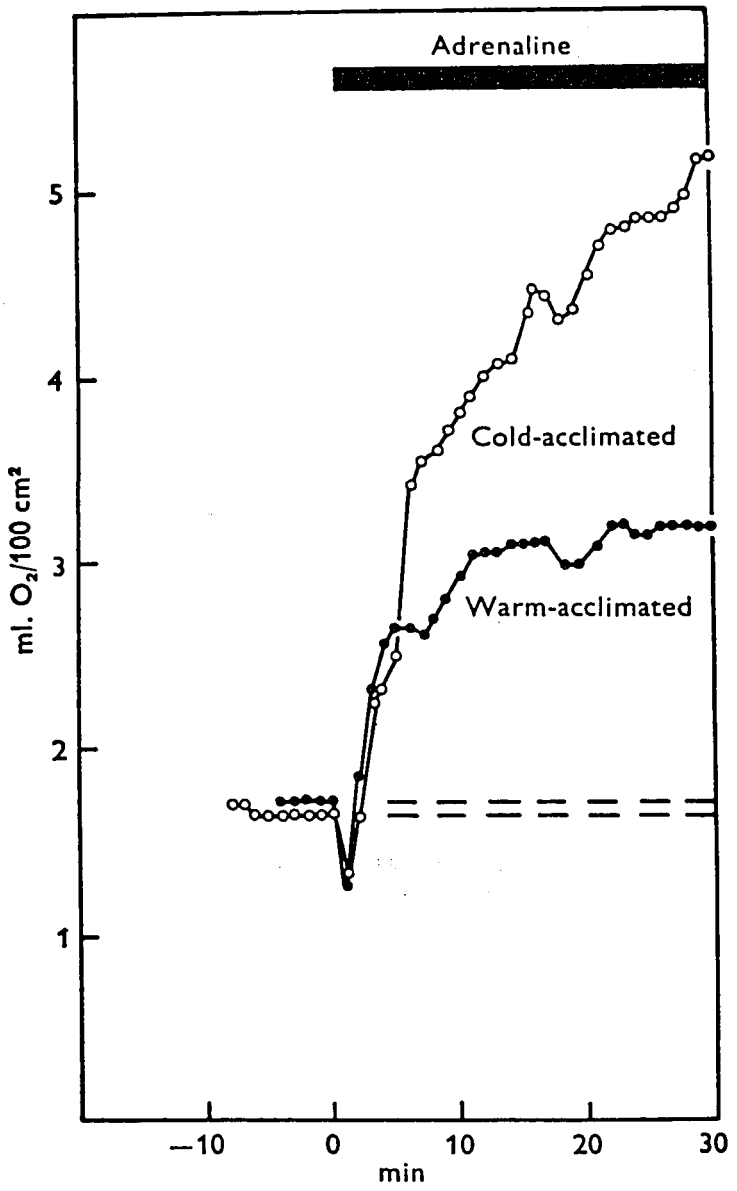

Fig. 5. Enhancement of calorigenic response to adrenaline by cold acclimation.

Oxygen uptake of a warm- and a cold-acclimated rat during infusion of adrenaline is shown. Adrenaline was infused intravenously (0.5 µg/100 cm^2 per min) from 0 to 30 min. Rats were lightly anesthetized with sodium pentobarbital. Warm-acclimated rat weighed 415 g; cold-acclimated rat weighed 331 g; they had lived at room temperature (25-28°C) and at 4°C, respectively, for 9 weeks. [From Himms-Hagen (86)].

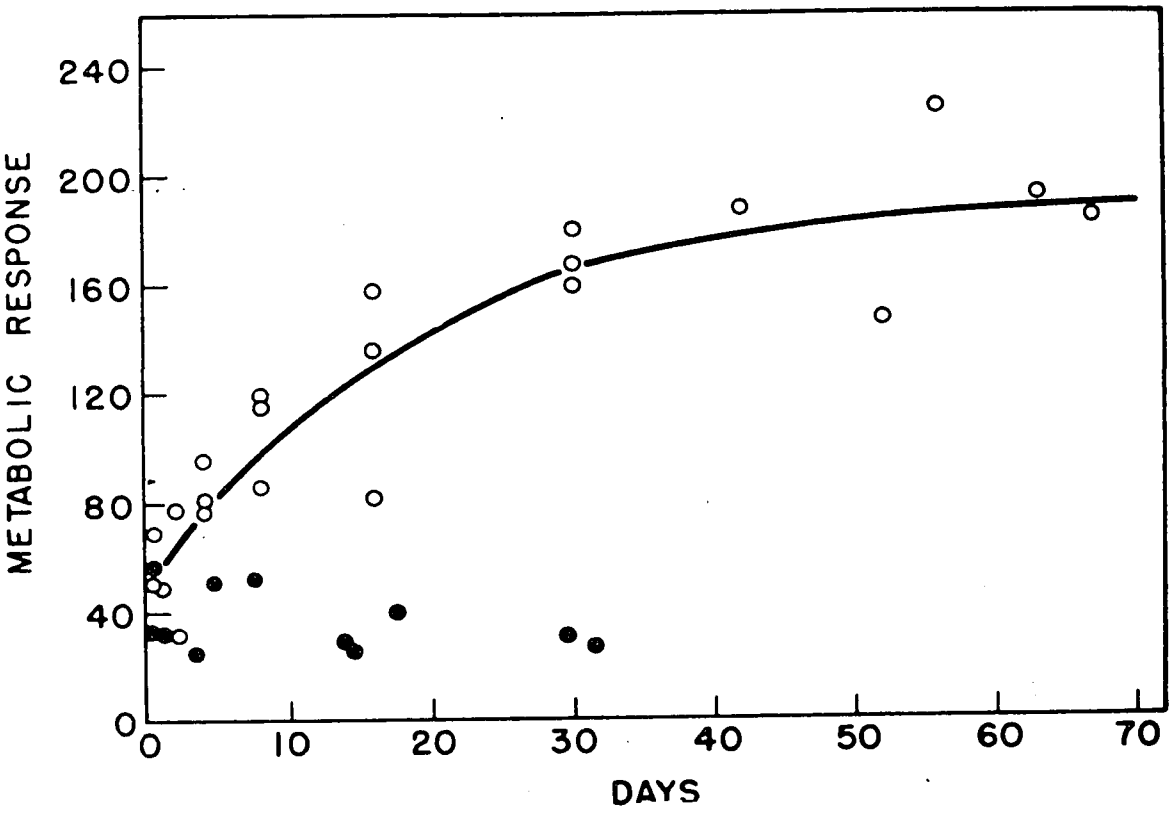

Fig. 6. Development of enhanced calorigenic response to noradrenaline during acclimation to cold.

Metabolic response to intravenously infused noradrenaline at level of 1 µg free base per min 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 (49)].

of time (134). They observed that the rats developed an adaptive state similar to that of cold-acclimated rats: the treated rats showed increased cold resistance and an enhanced response to noradrenaline.

In summary, these examples show that the mimicking effect of catecholamines occurs at two levels:

- switching on and off of NST (facultative process).
- development of cold acclimation (adaptive process).

The measurement of the calorigenic action of catecholamines can be used as an indicator of the capacity of an animal for NST.

C(iii). Inhibition of the sympathetic nervous system.

Immunological, pharmacological and surgical techniques have been used to inhibit the sympathetic nervous system, in order to determine its role in nonshivering thermogenesis. These techniques include adrenodemedullation, adrenalectomy, immunosympathectomy, the use of drugs such as reserpine, adrenergic neurone blocking agents (e.g. guanethidine), ganglionic blocking agents (e.g. chlorisondamine, mecamylamine) and inhibitors of catecholamine synthesis such as trimethyldopa and alpha-methyltyrosine. The inhibition of the sympathetic nervous system and its effects have been reviewed by Himms-Hagen (87). The general conclusion is that the sympathetic nervous system is directly involved in NST. However, the actual participation of the different parts of the sympathetic nervous system is difficult to define, due to the great adaptive capacity of the rat, i.e., when one

part of the sympathetic nervous system has been inhibited, some other regulatory mechanism tends to compensate for the perturbation caused by the inhibition.

D. Sites of nonshivering thermogenesis.

Which tissue is involved primarily in heat production in NST? The answer to this question has long been controversial. Different approaches have been taken to answer it:

- removal of the tissue to assess its contribution to the total oxygen consumption.
- cytochrome oxidase activity of the tissue.
- blood flow measurement together with measurement of arteriovenous differences in blood oxygen.

Evisceration of a cold-acclimated rat by occluding the blood supply to liver, intestines, stomach, spleen and pancreas did not seem to significantly affect the response to noradrenaline infusion (48,49). Surgical removal of interscapular brown adipose tissue (about one third of the total brown adipose tissue in the cold-acclimated rat) led to controversial results (see review by Himms-Hagen) (89): usually little effect just after removal of the tissue on the enhanced calorigenic response to noradrenaline in cold-acclimated rats (86) with progressive loss of the response during the four days following the operation. However, Foster obtained 14% decrease in the response one hour after removal of the interscapular brown adipose tissue. After four days, the calorigenic response to noradrenaline infusion was not significantly different from that of the

controls (57). Both these experimental approaches led to the conclusion that some tissue other than the viscera or brown adipose tissue must be the major site of NST.

Because of its large mass, it seemed probable that skeletal muscle would participate in NST. To assess its role, an indirect method was used: the values of cytochrome oxidase activity of homogenates appeared to be very similar to those of maximal steady state oxygen consumption measured in vivo. For this reason, Jansky measured the total cytochrome oxidase activity of the skeletal muscle mass. It was found to be 57% of the total (113). From values of cytochrome oxidase activity in brown adipose tissue, the conclusions of Jansky and coworkers pointed to muscle as the major site of NST (117).

Jansky and Hart (116) measured blood flow to various organs with Sapirstein's technique based on distribution of radioactive rubidium (201). They found that brown adipose tissue would contribute to only 6% of NST in the cold-acclimated rat and that muscle contribution could not exceed 50% of the total (116).

The oxygen consumption of the partly isolated leg muscles was measured in situ in cold-acclimated rats before and during exposure to cold (115). Oxygen consumption approximately doubled during cold-exposure and noradrenaline infusion (Fig. 7) (115) but without any increase in blood flow. A 49% increase in oxygen consumption was reported in

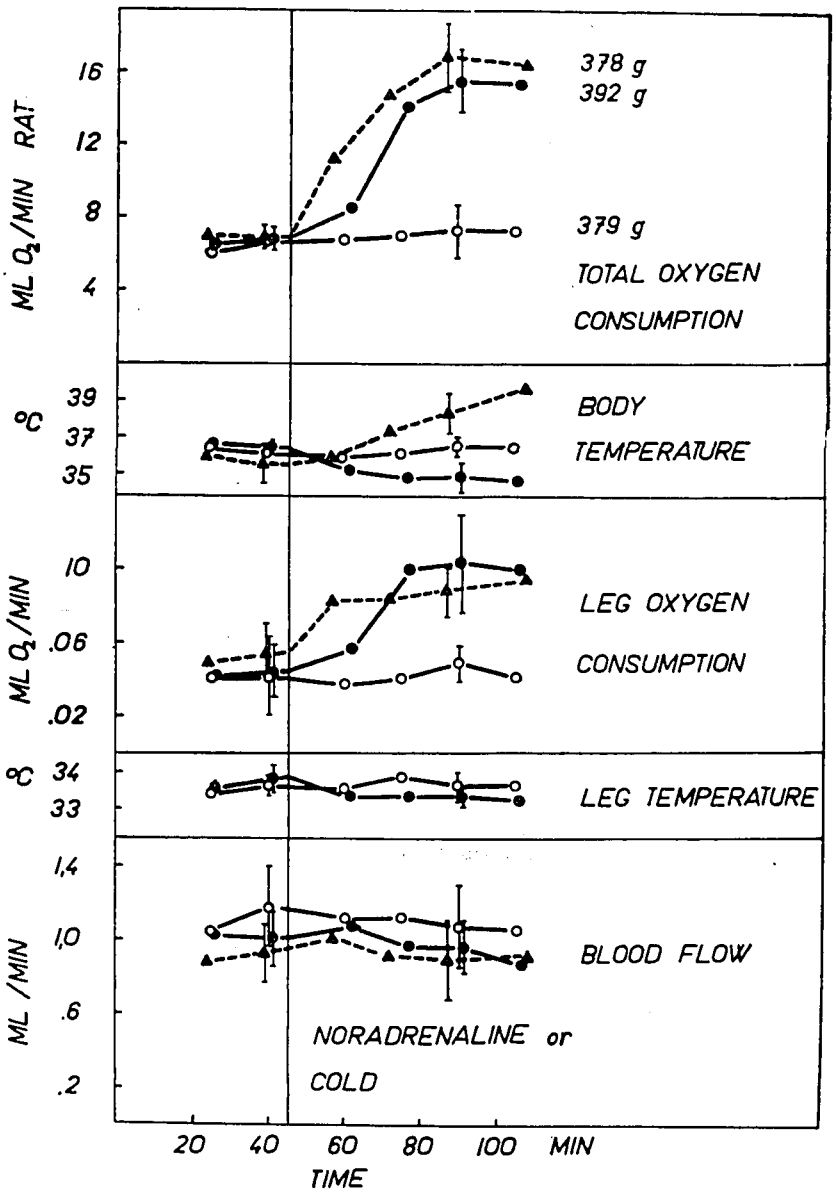

Fig. 7. Occurrence of nonshivering thermogenesis and of calorigenic response to noradrenaline in leg muscle of cold-acclimated rats.

Comparison of responses in the leg and in the whole rat before and during noradrenaline infusion at 0.8 µg/min (▲) or exposure to 10°C (●). Vertical line at 45 min shows start of noradrenaline or cold. Untreated controls (○) are also indicated. Vertical lines denote standard deviation. Results not corrected for change in body temperature during tests. [From Jansky & Hart (115)].

the isolated gracilis muscle of the cold-acclimated rat during noradrenaline infusion (Fig. 8), but no control values for the warm-acclimated rat were given (165). More recently, a similar study was done on isolated perfused rat muscle. Although the increase of the oxygen consumption in response to noradrenaline was initially the same in cold-acclimated rats and control rats, the limbs from the cold-acclimated rats were able to maintain the elevated steady state oxygen consumption during 30 minutes whereas for control rats, a decrease was observed (Fig. 9) (75).

It appears that the contribution of skeletal muscle to NST is difficult to assess, especially because the different muscles do not contribute the same way and extrapolation from one muscle to the whole muscle mass might be incorrect. Another major problem with muscle is the fact that the individual muscle fibers do not possess sympathetic innervation. However, the response to noradrenaline infusion in vivo does exist (75, 116, 165).

Thus, until approximately 1977, it was generally believed that muscle was the major site of NST, despite the difficulty in obtaining a quantitative estimate of its contribution. However, recently reported studies of Foster and Frydman (58, 59) indicate that previous estimates of blood flow in which radioactive rubidium uptake was measured are invalid because brown adipose tissue does not take up this ion in proportion to its blood flow. By using radioac-

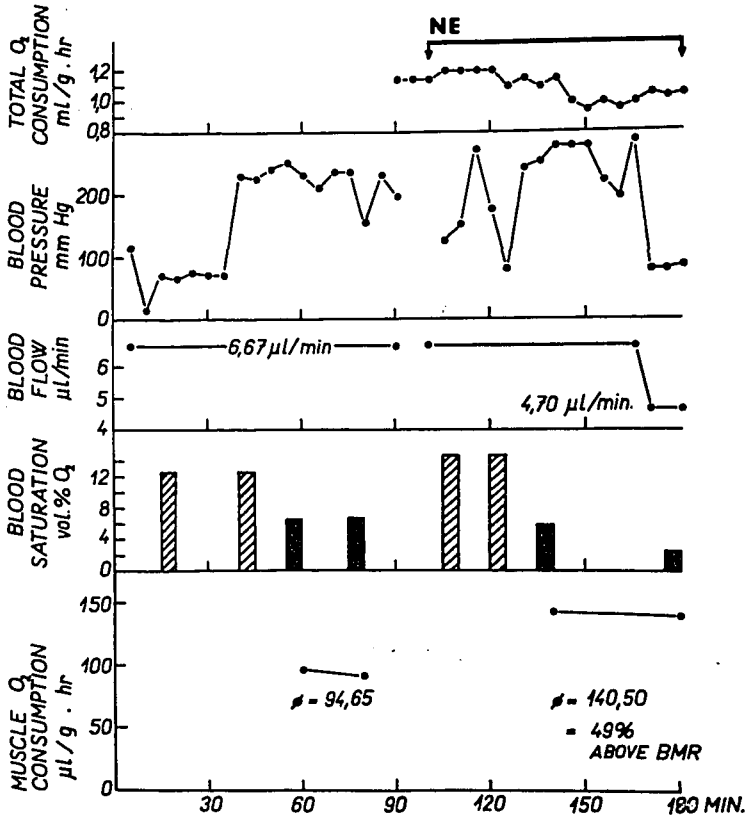

Fig. 8. Oxygen consumption in the isolated gracilis muscle of cold-acclimated rats during noradrenaline infusion.

The lower tracing shows the oxygen consumption of gracilis muscle perfused in situ, before (left) and during (right) infusion of noradrenaline. [From Mejsnar & Jansky (165)].

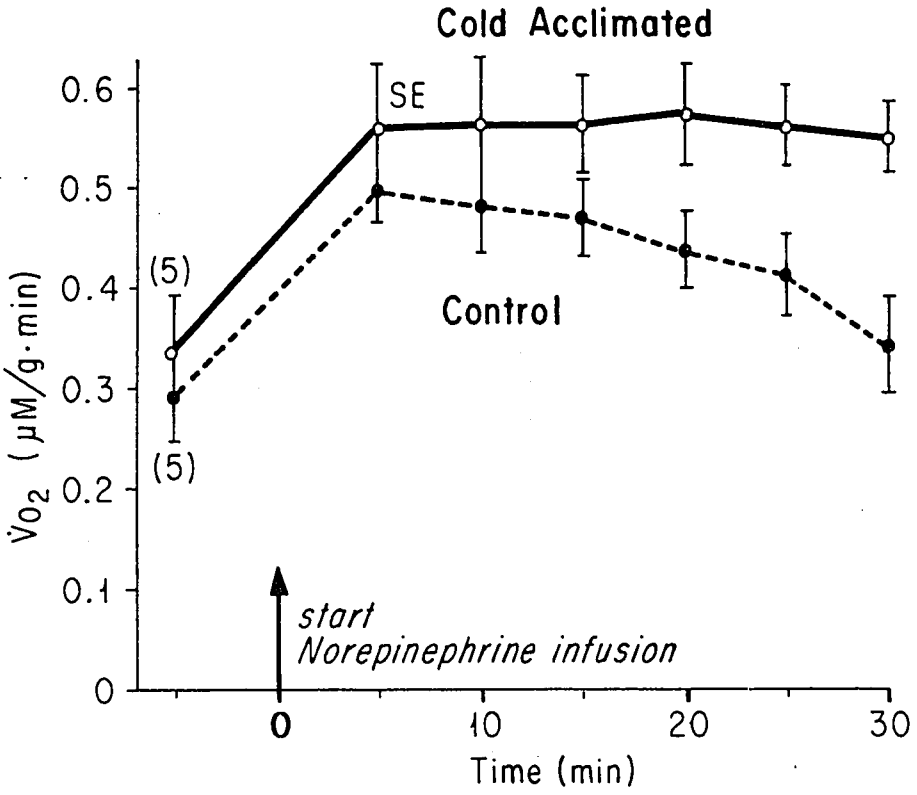

Fig. 9. Time-course of the increase in the rate of oxygen consumption of the rat hindlimb during noradrenaline infusion.

The graph shows the oxygen consumption $\dot{V}_{O_2}$ of perfused muscle from the cold-acclimated rat (o) and the warm-acclimated rat (•) during a 30 min infusion of noradrenaline (0.01 μg/g. min). [From Grubb & Folk (75)].

tive microspheres, they have found a very high increase in the blood flow in brown adipose tissue when the cold-acclimated rat is infused with noradrenaline (2.3 ml/min to 57.2 ml/min); the increase in percentage of cardiac output follows the same pattern (2.6% at rest vs 33.5% during noradrenaline infusion). For muscle, they observed a much smaller increase in blow flow (9.9 ml/min to 14.5 ml/min) and a decrease in the percentage of cardiac output (from 11.2% to 8.4%). From their results, Foster and Frydman estimate the contribution of brown adipose tissue to be 60% of NST, leaving about 12% for muscle. Moreover, they propose that the 14% reduction in the response of cold-acclimated rats to noradrenaline observed after removal of the interscapular brown adipose tissue (one fourth of the total in this experiment) (57) was the actual contribution of the tissue to NST. They suggest that the failure to find a persistent reduction in the calorigenic response after four days is due to compensation by other masses of brown adipose tissue (59).

In conclusion, recent evidence suggest that brown adipose tissue is the major site of nonshivering thermogenis. However, skeletal muscles from cold-acclimated rats are capable of responding to noradrenaline by an increase in oxygen uptake to a greater extent than those of warm-acclimated rats (75) and probably contribute to some extent to NST. Moreover, they undergo changes during cold-acclimation

(see section II.2.c.ii).

In summary, rats, among other species, are able to adapt to a cold environment. The mechanism which produces the heat is called nonshivering thermogenesis. The switching on and off of NST is mediated by the sympathetic nervous system. The major sites of NST appear to be brown adipose tissue and skeletal muscle.

PART 2 : BIOCHEMICAL MECHANISMS OF NONSHIVERING THERMOGENESIS.

In this section, the biochemical aspects of heat production will be considered. First, the general concepts of intracellular thermogenesis as well as its control will be reviewed, then the various hypotheses which could explain the mechanism of NST, and finally, the adaptive changes which have been observed in association with acclimation to cold.

A. General mechanisms of thermogenesis.

The principal function of the oxidation of foodstuffs (glucose, fatty acids, amino acids) by animal cells is the production of ATP which is the major form of energy in the cell. However, the combustion of fuel produces heat as a by-product. The site of heat production has not been defined clearly. According to Prusiner and Poe (190, 191), the mitochondrion would be the major site of heat production with electron transport and oxidative phosphorylation the major heat producing process.

Two major theories exist concerning the control of heat production. In the first one, the rate of respiration is regulated by the phosphorylation state ratio $[ATP]/[ADP][P_i]$ (182,238), which means that in the mitochondrion, the respiration is fully coupled to oxidative phosphorylation and that substrates as well as oxygen are not limiting. The second theory is based on uncoupling or rather loose-coupling of the mitochondrion. Uncoupled mitochondria respire at maximal rate but are incapable of phosphorylating ADP. What limits the respiration rate is the substrate availability. In loosely-coupled mitochondria, oxidative phosphorylation occurs but does not control the rate of respiration. With fully coupled mitochondria, what regulates the heat production is any process which utilizes ATP and thus decreases the phosphorylation state ratio; for example, specific ATPases such as Na,K-ATPase, myosin ATPase, Ca-ATPase or any futile cycle made of a synthetic pathway, in which ATP is used, coupled with a catabolic pathway (e.g. triglyceride cycle). The state of coupling and thus the rate of respiration are regulated by several factors including free fatty acids, nucleotides, thyroid hormones, histones. The role of these factors as well as the various ATP-utilizing processes have been reviewed by Himms-Hagen (89) in the description of examples of cellular thermogenesis. This review will be limited to one thermogenic process, namely nonshivering thermogenesis.

B. Hypotheses for the mechanism of nonshivering thermogenesis.

There are two main hypotheses to explain the biochemical mechanisms of NST : the loose-coupling hypothesis and the Na,K-ATPase hypothesis. Whereas the first one can be considered only for brown adipose tissue, the second can be applied to both muscle and brown adipose tissue. As NST is a calorigenic response to catecholamines, these hormones must play an important role in both hypotheses.

B(i). Loose-coupling hypothesis.

In the chemiosmotic theory proposed by Mitchell (167), the synthesis of ATP is linked to the electron transport chain by a circuit of protons. The synthesis of ATP is driven by the proton electrochemical gradient generated by the expulsion of protons from the mitochondrion during electron transport. In the coupled mitochondria, when ADP is absent, proton re-entry ceases. This leads to a build-up in the proton electro-chemical gradient preventing further proton release by the respiratory chain. However, if protons can re-enter the matrix by an alternative pathway not linked to the synthesis of ATP, respiration can still occur. In this case, the mitochondrion is said to be loosely coupled.

Brown adipose tissue mitochondria appear to be very sensitive to the extraction conditions. When they are extracted by conventional methods, they show rapid rates of respiration, low P/O ratios and lack of respiratory control:

they are loosely coupled (56). However, they can be recoupled by a variety of means, including addition of bovine serum albumin (72) usually together with nucleoside di- and triphosphates (27,211), carnitine and ATP, suggesting that free fatty acids are responsible for the uncoupling (92). Free fatty acids seem to play a dual role in brown adipose tissue mitochondria: fuel as well as uncoupling agent.

Fairly recently, an additional pathway for proton re-entry, independent of ATP synthesis, has been found in brown adipose tissue mitochondria (178). Proton re-entry is regulated by purine nucleotides which bind to a specific site on the outer face of the mitochondria inner membrane (176,177). Binding of nucleotides is sensitive to pH (39), and regulated by fatty acyl CoA molecules such as palmitoyl CoA (28). The nucleotide-sensitive proton conductance pathway provides a good explanation of the uncoupling mechanism. However, the hormonal control remains to be clarified. One hypothetical description of the chain of events is the following: noradrenaline stimulates adenylate cyclase which catalyzes the formation of cyclic AMP. Cyclic AMP in turn induces lipolysis through a protein kinase. The fatty acids (79) or fatty acyl CoA (28) liberated displace purine nucleotides and make the mitochondria loosely coupled by increasing the proton conductance. When the stimulus is removed, the uncoupler level gradually decreases and the mitochondria are recoupled after binding of purine nucleotides. There are

however some difficulties whith this hypothesis: no significant changes in intracellular (20) or intramitochondrial (184) fatty acid level have been observed during noradrenaline-stimulated brown adipose tissue respiration. Purine nucleotide concentrations do not change significantly on noradrenaline addition either (186). Moreover, beta-receptor antagonists inhibit cellular respiration almost instantaneously without depleting cellular fatty acids (186).

In conclusion, one can see that there is a gap in the chain of events as it has been described: a messenger between the plasma membrane and the mitochondrion remains to be found to explain how the short-circuit is switched on upon noradrenaline addition and off when noradrenaline is no longer present.

B(ii). Na,K-ATPase hypothesis.

The second main hypothesis to explain the mechanism of NST involves plasma membrane rather than mitochondria and can be proposed for muscle as well as brown adipose tissue.

This hypothesis has been proposed by Horwitz (96) for brown adipose tissue. It derives from Edelman's hypothesis of the role of Na,K-ATPase in thyroid thermogenesis (see section II. 3.A.ii.). In this hypothesis, the noradrenaline-induced increase in oxygen consumption (and heat production) is due to an increased activity of the Na,K-ATPase located on the plasma membrane. The involvement of the Na,K-ATPase

was first suggested by Girardier et al., (65) following their observation of noradrenaline-induced depolarization of brown adipose tissue cells. This observation has been confirmed in vitro (54,132,236) as well as in vivo, after electrical stimulation of the nerves innervating the interscapular brown adipose tissue (101), or after intravenous injection of noradrenaline (55,101), isoproterenol or phenylephrine (55). That the thermogenic response to noradrenaline is associated with an enhanced permeability of the membrane to ions such as Na^+ and K^+ is shown by several lines of evidence: increased intracellular Na^+ and decreased intracellular K^+ concentrations (65), increase in brown fat temperature (101) or oxygen consumption (208) occurring after the redistribution of ions across the adipocyte membrane and finally, altered membrane resistance (95). After the stimulus has been removed, restoration of ion distribution has been observed in vivo (55,101) as well as in vitro (65,208). A direct stimulation of the Na,K-ATPase by noradrenaline, through cyclic AMP, has been shown on membrane fractions isolated from brown adipose tissue of cold-acclimated rats (82,98). The contribution of Na,K-ATPase to respiration is assessed by experiments based on sodium pump blockade either by ouabain, a specific inhibitor of the Na,K-ATPase or by lack of sodium. For example, a 60% decrease in the noradrenaline-induced respiratory response has been found in isolated brown fat cells of hamster (96). The oxygen consumption elicited by either isoproterenol or phenylephrine is decreased by 70% in the presence of ouabain

(97). However, Chinet and coworkers (38) measured the energy expenditure due to active sodium-potassium transport in the brown adipose tissue of rat; the contribution of Na,K-ATPase (assessed by ouabain inhibition) to the total heat production seemed to be of little importance.

It appears that the use of ouabain to assess the role of Na,K-ATPase in noradrenaline-induced thermogenesis in brown adipose tissue can lead to erroneous conclusions (see 89): ouabain also inhibits the stimulation of adenylate cyclase by noradrenaline as well as the lipolytic response to noradrenaline. Fatty acid production is also inhibited. The role of fatty acids in the loose-coupling hypothesis has been outlined above; thus, it appears that the use of ouabain might not be helpful in distinguishing between the two hypotheses. Moreover, studies by Cannon and Vogel (29) indicate that brown adipose tissue mitochondria have five times more respiratory-chain components and less ATPase per mg protein than liver mitochondria. These data indicate a small capacity of brown adipose tissue for oxidative phosphorylation as well as a great capacity for uncoupled respiration. In addition, this hypothesis is not compatible with the acyl CoA-induced inhibition of the adenine nucleotide translocation reported by Christiansen and coworkers(40).

In muscle, the hypothesis concerning the role of Na,K-ATPase in heat production has been proposed by Stevens (218). An increased ouabain-sensitive oxygen uptake has

been found in muscle slices from cold-acclimated mice (219), indicating that this part of the oxygen consumption might be due to operation of the Na,K-ATPase. Other experiments led to similar results: an increase in respiration rate in the diaphragm of cold-exposed and cold-acclimated rats has been observed (168), which was inhibited by ouabain or replacement of Na^+ by Li^+ in the incubation medium. A similar increase in ouabain-sensitive respiration has been obtained with diaphragm muscle from cold-acclimated hamsters (99) and diaphragm and pectoral muscles from cold-acclimated rats (76).

These examples suggest a possible role of catecholamines in the regulation of Na,K-ATPase activity. In anesthetized hamsters, intravenous injection of noradrenaline was followed by a decrease in potential across the sarcolemma of the sartorius and gracilis anticus muscles (227). Thus, noradrenaline appears to promote ion changes. Moreover, catecholamines have been found to stimulate Na^+-K^+ transport in rat muscles (41,198).

In spite of these data, no relationship has been established between Na,K-ATPase increased activity and muscle nonshivering thermogenesis. An apparently permanent increase is observed in the activity of the sodium pump, although the basal metabolic rate of both cold-acclimated and warm-acclimated animals is the same in a warm environment. Moreover, similar change occurs in both the cold-acclimated animal and

the cold-exposed animal (168) although cold acclimation is an adaptive process which takes four weeks to be achieved. The initial response to noradrenaline infusion in the isolated hindlimb has the same magnitude in the cold-acclimated rat and the warm-acclimated rat (see Fig. 9) (75).

Thus, the mechanism of NST in skeletal muscle is uncertain and requires further investigation.

C. Adaptive changes known to occur in association with altered nonshivering thermogenesis.

A good approach in trying to determine the mechanism of NST is to compare the biochemical characteristics of the organs involved in NST from warm-acclimated animals with those of cold-acclimated animals. One can expect any change to reflect the increased capacity to use NST. The following section is a review of the main changes observed in rat brown adipose tissue and skeletal muscle during and after acclimation to cold.

C(i). Brown adipose tissue.

Brown adipose tissue is an organ found in homeotherms; its main role is heat production. It is abundant in hibernators, certain cold-acclimated species as well as newborn mammals. Brown adipose tissue has a rich sympathetic innervation and blood supply. The cells are packed with mitochondria and lipid droplets. The mitochondria are generally elongated with numerous parallel-oriented cristae. The high respiration of the tissue is explained by a high proportion

of inner membrane, hence high concentration of respiratory enzymes (29, 189).

During acclimation to cold, brown adipose tissue grows; the mitochondrial mass increases as well as the respiratory capacity of mitochondria. The rate of state four respiration (in the absence of ADP) is increased and becomes equal to the rate of uncoupled respiration (179), meaning that the mitochondria are loosely coupled. As already mentioned (II.2.B.i.), loosely-coupled mitochondria can be recoupled by purine nucleotides. The capacity to bind purine nucleotides is increased with increased capacity for NST (Fig. 10) (51). The binding site for purine nucleotides has been recently identified in hamster and guinea pig brown adipose tissue mitochondria: it is a 32 000 polypeptide located on the inner membrane of the mitochondrion (80). It appears that there is a large increase in the amount of a 32 000 polypeptide in brown adipose tissue mitochondria from cold-acclimated rats (91,192). This increase and the increased capacity to bind purine nucleotides can be associated with the increased extent of loose-coupling observed in mitochondria from cold-acclimated rats (179).

Thus, brown adipose tissue mitochondria show qualitative as well as quantitative changes during acclimation to cold.

At the level of the plasma membrane, little information is available. A reduction (169) or no change (212) in the

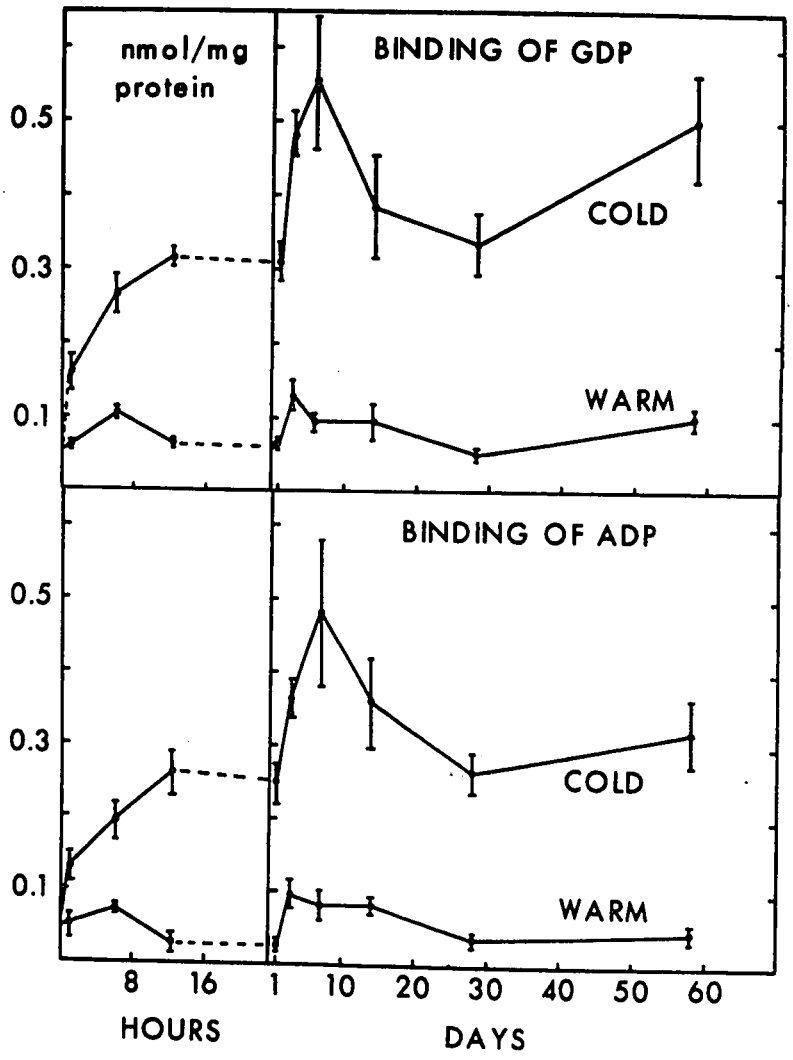

Fig. 10. Binding of GDP and ADP by brown adipose tissue mitochondria during acclimation to cold.

The graphs compare the binding during acclimation to cold (COLD) with the binding in control rats (WARM). The left-hand part of each section of the figure shows changes during the first day and the right-hand part shows changes during the subsequent 8 weeks. Values are means (± SEM) of three observations. [From Desautels et al., (51)].

catecholamine-stimulated adenylate cyclase activity has been observed. A 41% decrease in the density of beta-adrenergic receptors has been reported by Bukowiecki et al., (25).

C(ii). Skeletal muscle.

Skeletal muscle mitochondria have been studied in some detail. They appear different in the cold-acclimated rat at various levels. They are smaller and more numerous (17). In contrast to brown adipose tissue mitochondria, the capacity to bind purine nucleotides is extremely low and unchanged by acclimation to cold (Desautels & Himms-Hagen, unpublished results). These data correlate with the absence of the 32 000 polypeptide as well as with unchanged ADP/O or respiratory control ratios (90). However, the rates of ADP-stimulated (90) and calcium-stimulated (74) respiration are increased, changes apparently due to an increased adenine nucleotide translocase activity (Desautels & Himms-Hagen, unpublished results) and to an increased rate of calcium transport (74) although calcium content is unchanged. These changes suggest an increased capacity for coupled respiration.

At the sarcolemma level, no change occurs in the catecholamine-stimulated adenylate cyclase (170).

In conclusion, the changes observed in brown adipose tissue and skeletal muscle of cold-acclimated rats favor the loose-coupling hypothesis for brown adipose tissue (increased

rate of state four respiration plus increased binding of nucleotides) and an ATP-consuming process hypothesis like the Na,K-ATPase hypothesis for muscle (increased rate of ADP-stimulated respiration).

PART 3 : CHARACTERISTICS OF PLASMA MEMBRANES.

In this section, the characteristics of plasma membranes will be considered on a functional basis only. What is known about Na,K-ATPase, beta-adrenergic receptors and adenylate cyclase will be reviewed. The important role of catecholamines in nonshivering thermogenesis has been demonstrated (see section II.1.C.); for this reason, in this review, the possible regulation by catecholamines of the plasma membrane components mentioned above will be stressed.

A. $(Na^+ + K^+)$ activated ATPase.

The possible role of the Na,K-ATPase in the mechanism of nonshivering thermogenesis has already been considered (see section II.2.B.ii.). In the following section, general characteristics of the enzyme will be reviewed. Then, Edelman's hypothesis of thyroid thermogenesis, which is based entirely on changes in Na,K-ATPase activity, will be described in some detail. Finally, some aspects of the action of hormones, especially catecholamines, will be considered. This review will permit an understanding of how

the response of the cold-acclimated rat to catecholamines could be mediated through a change in Na,K-ATPase activity.

A(i). General considerations concerning the Na,K-ATPase.

In 1957, Skou found a Mg^{++}-dependent ATPase in the microsomal fraction of leg nerves from the shore crab. The enzyme was activated by the presence of Na^+ and this activation was enhanced if K^+ was also present (213). Skou suggested that this enzyme might be involved in Na^+ transport. Since then, several lines of evidence have developed, showing the identity of this enzyme, referred to as the (Na^+ + K^+)activated ATPase (Na,K-ATPase), and the sodium pump. For example, high transport activity in tissues is parallel with high Na,K-ATPase activity; both transport and Na,K-ATPase require ATP and Na^+ on the inside and K^+ on the outside of the membrane. Ouabain inhibits both enzyme and transport by binding on the outer face of the membrane (see 93).

The Na,K-ATPase appears to be confined to plasma membranes: with an immunologic technique, Kamat and Wallach (123), working with Ehrlich ascites carcinoma cells, isolated, on a density gradient, 67% of Na,K-ATPase activity together with 88% of cell surface antigen. Barclay and coworkers (11) found a twelve-fold increase in Na,K-ATPase activity in rat liver plasma membrane compared to homogenate.

Although ATP is the preferred substrate for the enzyme, other nucleotides can also be used. Relative activi-

ties of 100 : 49 : 2.3 ::2.4 : 0.6 have been reported for ATP, d-ATP, CTP, ITP and GTP respectively (81).

Na^+ and K^+ are both required to activate the Na,K-ATPase but high concentrations of either one inhibit the activation by the other. Several other ions can replace K^+ with the following order of effectiveness: $K^+ > Rb^+ > NH_4^+ > Cs^+ > Li^+$ (214). Thallium can also substitute for K^+ with an affinity ten times higher (23).

The stoichiometry for the erythrocyte Na,K-ATPase appears to be three Na^+ pumped outward and two K^+ pumped inward for every ATP hydrolysed (93). Using K^+-rich resealed erythrocyte ghosts incubated in a Na^+-rich medium a reversal of the pump, coupled to synthesis of ATP, was observed (64). Other exchanges have been reported; for example, an efflux of Na^+ coupled to an influx of Na^+ (9,68), which requires ATP as well as ADP (34). A K^+ efflux coupled to a K^+ influx has also been shown (69).

The Na,K-ATPase hydrolyses ATP in a stepwise fashion which involves a Na^+-dependent phosphorylation of the enzyme and a K^+-dependent dephosphorylation. The reaction sequence is described in Figure 11. The first step involves the formation of an ATP-enzyme complex. The presence of Mg^{++} is not required for ATP binding but if the enzyme is treated with ouabain or K^+, binding of ATP does not occur (81). Na^+ antagonizes the inhibition by K^+ but has no direct effect on ATP binding. ADP is effective in displacing ATP from its binding site (81). The existence of a phosphorylated intermediate

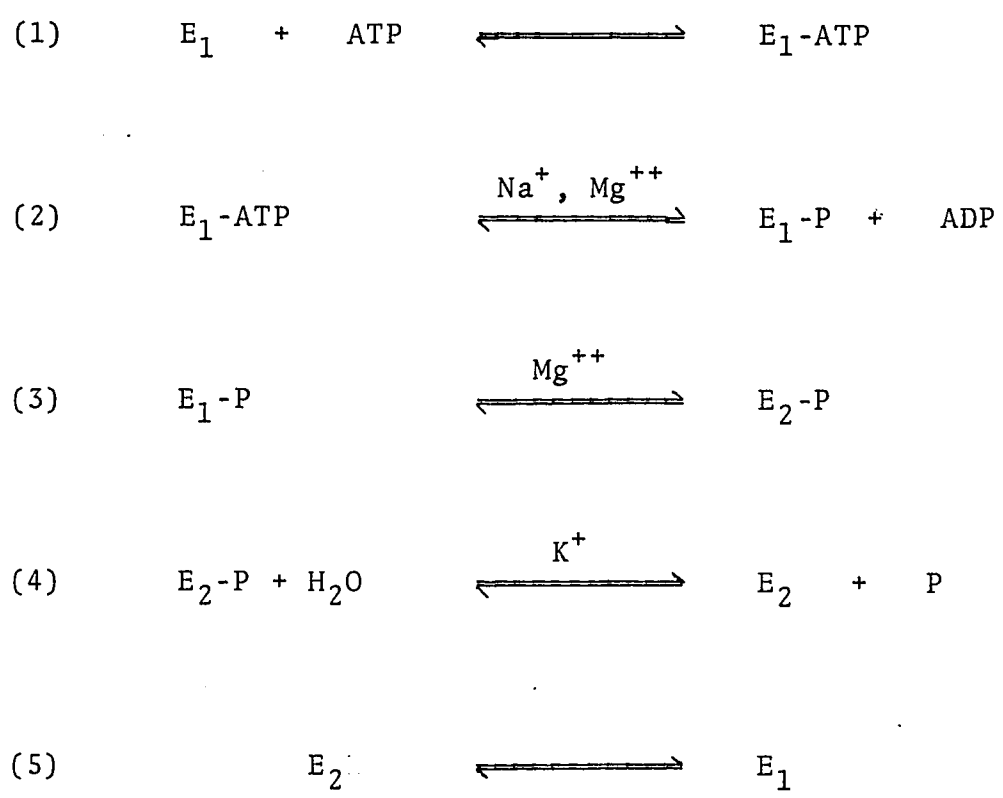

Fig. 11. Na,K-ATPase : reaction sequence

(1) Binding of ATP.
(2) Phosphorylation of the enzyme. (Na^+-dependent).
(3) Conformational change of the phosphorylated intermediate.
(4) Dephosphorylation of the enzyme. (K^+-dependent).
(5) Conformational change of the enzyme.

[From Dahl & Hokin (46)].

was demonstrated by the finding of a Na^+-dependent incorporation of ^{32}P from [α-^{32}P]ATP into Na,K-ATPase preparations (see 46). The simultaneous presence of K^+ greatly reduced the radioactivity recovered. Addition of K^+ after Na^+ discharged ^{32}P rapidly, indicating a K^+-dependent dephosphorylation. This dephosphorylation was inhibited by low concentrations of ouabain whereas high concentrations prevented phosphorylation itself (205). Na,K-ATPase preparations exhibit K^+-dependent phosphatase activity corresponding to step (4) in Fig. 11. This step is ouabain inhibitable. There is a parallel enrichment of the two activities on purification of the Na,K-ATPase (46).

The Na,K-ATPase is inhibited by cardiotonic steroids such as ouabain. The first observation of this specific inhibition was made only three years after Skou's discovery of the enzyme (188). Since then, a considerable literature has accumulated on the effects of cardiotonic steroids on Na^+ and K^+ transport as well as their mechanism of action (see 8,93). Cardiotonic steroids have the ability to exert a powerful action on the heart on injection into man or animal. They have been used extensively in the treatment of cardiac disorders for over two hundred years. Low concentrations of cardiotonic steroids have a therapeutic effect but high concentrations are toxic. The highly specific nature of the inhibition of Na,K-ATPase by cardiotonic steroids has led to the suggestion that the enzyme is the pharmacologic recep-

tor for these compounds, their action on the enzyme being responsible for their therapeutic and toxic effects. Cardiotonic steroids inhibit the enzyme by binding to the outer face of the membrane. Binding is favored by the presence of Mg^{++}, Na^{+} and ATP, thus, by conditions that favor the formation of the phosphorylated intermediate (see Fig. 11). K^{+} competes with low concentrations of cardiotonic steroids (93).

Na,K-ATPase is an intrinsic component of the cell membrane. This fact explains why the enzyme is difficult to purify. The common sources used for purification are brain and kidney (especially the outer medulla) which are very rich in Na, K-ATPase; but the richest sources are organs which are specialized for Na^{+} and K^{+} pumping, such as the electric organ of Electrophorus electricus (3) and the rectal gland of Squalus acanthias (94). Purification of the Na,K-ATPase requires solubilization by detergents which may lead to inactivation of the enzyme (see 121). Specific activities in the range of 1200 to 1500 µmoles P_i/mg protein per hour have been reported after purification (see 46). SDS-polyacrylamide gel electrophoresis of the purified Na,K-ATPase indicates the presence of two major polypeptides. The larger one has been reported to have a molecular weight between 84 000 and 100 000 (46) and is the polypeptide which is phosphorylated during the course of ATP hydrolysis (see above). The second one, a glycoprotein, has a molecular

weight in the range of 47 000 to 56 000 as estimated by SDS gel electrophoresis (46). Purified preparations having 60-70% of the large polypeptide and 3-4 nmoles of g-strophanthin (ouabain) binding sites per mg protein, show very variable specific activities (215). This might be due to species differences but another possibility is that the lipid environment is different in the preparations obtained with a variety of means.

Indeed, lipids play an important role in Na,K-ATPase activity. The general agreement is that lipid-depleted preparations (by detergents, solvents or phospholipases) can be reactivated by addition of phospholipids. Although phosphatidylserine (PS) seems responsible for the reactivation in a number of cases (71,128,197,225), activation by phosphatidylinositol (PI) (225) and phosphatidylcholine (PC) (84) has been reported. More recent findings however (183), indicate that specificity in lipid requirement is not absolute, as long as the lamellar organization with appropriate density of negative charges is present around the protein components.

A(ii). Role of Na,K-ATPase in respiration and thermogenesis.

The role of cation transport in control of respiration was first proposed by Whittam in 1961. He observed a 70% decrease in K^+ concentration together with a 50% decrease in QO_2 after incubating brain slices with ouabain. The phemenon was also observed when Na^+ was replaced by choline chlo-

ride, and no further decrease occurred upon ouabain addition. Similar results were obtained with kidney slices (234). Since that time, the role of Na,K-ATPase in the control of respiration has been widely studied (230,235). Apart from a possible involvement in nonshivering thermogenesis (see section II.2.B.ii.), Na,K-ATPase has been proposed to play a role in the evolution of endothermy (218). More recently, a 50% reduction of the Na,K-ATPase activity in skeletal muscle from obese mice has been proposed as an explanation of the lower body temperature, the reduced heat production, and lower maintenance energy requirement observed in these animals (152).

The concept of control of respiration by Na,K-ATPase is, in particular, the basis of the hypothesis for the mode of action of thyroid hormones put forward by Edelman. In one of the first experiments, the oxygen consumption of liver slices and diaphragm from thyroxine- and triiodothyronine-injected thyroidectomized and normal rats were measured (106). Sodium pump contribution to oxygen consumption was assessed by the presence of ouabain or the absence of Na^+ or K^+ in the incubation medium. In liver as well as diaphragm, more than 90% of the increase in QO_2 produced by injections of thyroid hormones in euthyroid rats was elicited by increased activity of the sodium pump. In triiodothyronine-treated thyroidectomized rats, increased energy utilization by the sodium pump was responsible for 90% of the increase in QO_2 in liver and 40% in diaphragm (106). These

results were confirmed by measurements of Na,K-ATPase activity. The triiodothyronine-induced increase in Na,K-ATPase activity paralleled the increase in oxygen consumption in liver and kidney of thyroidectomized and euthyroid rats. In addition, the authors observed no change in either respiration or Na,K-ATPase of brain slices (107).

Thyroid hormone-dependent activation of the sodium pump could be a result of four different mechanisms of action (see Fig. 12) (52).

1 Increased passive permeability to Na^+ and K^+: Na^+ concentration inside the cell rises and activates the sodium pump.

2 Uncoupling of the sodium pump: more ATP is used for the same transport of Na^+ and K^+

3 Increase in the synthesis of ATP, the other substrate.

4 Direct activation or unmasking of preexisting sodium pumps or synthesis of new sodium pumps.

Mechanisms 1 and 2 should lead to an increase or, at least, no change in the intracellular Na^+/K^+ ratio whereas mechanisms 3 and 4 should cause a decrease in this ratio. Ismael-Beigi and Edelman reported a 20 to 40% decrease in the Na^+/K^+ ratio in liver slices, diaphragm and heart after administration of triiodothyronine to hypothyroid and euthyroid rats. Thus, hypotheses 1 and 2 could be eliminated. A good way to

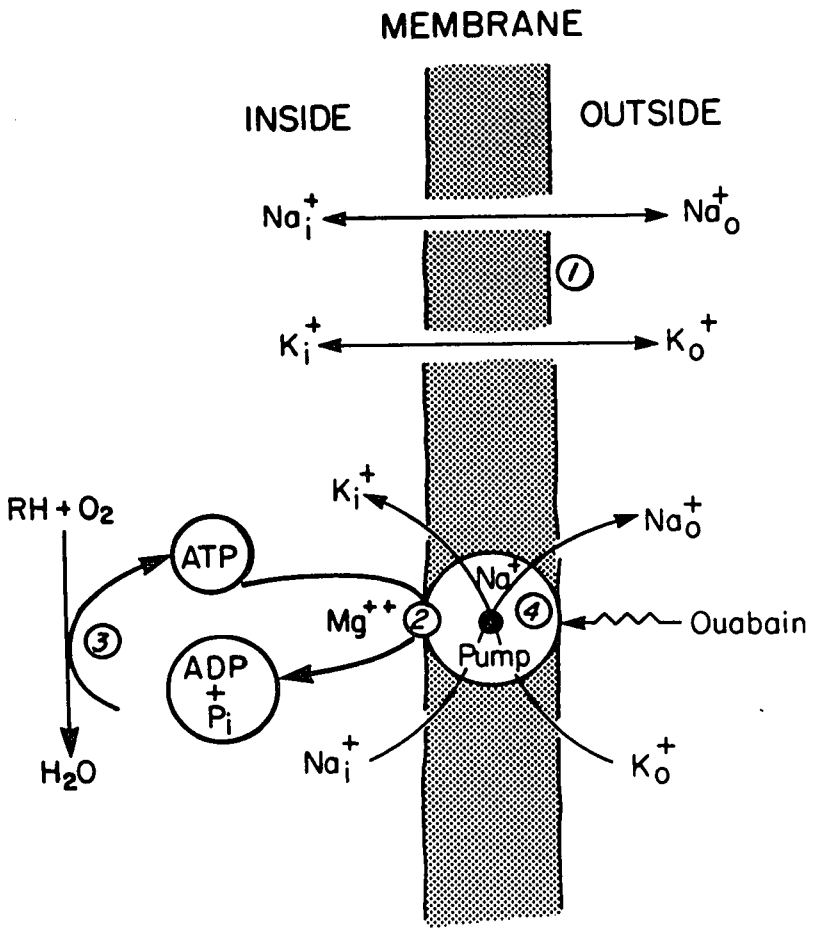

Fig. 12. Sites of regulation of Na^+ transport across cell membrane.

Site 1 indicates passive Na^+ and K^+ permeability channels. Site 2 is the coupling of ATP hydrolysis to active Na^+ transport (or Na^+:K^+ linked transport). Site 3 designates the regeneration of ATP coupled to oxidation of reduced substrates (e.g. NADH, succinate). Site 4 indicates actions on the Na^+ pump either by stimulation of a set of pre-existing pumps or by increasing the number of pumps. [From Edelman (52)].

distinguish between hypotheses 3 and 4 was to measure the ATP/ADP ratio. In case of mechanism 3, an increase of this ratio should occur whereas a decrease should be observed if mechanism 4 predominates. Nucleotide content of euthyroid and thyroidectomized rat liver was determined using a rapid freeze technique in situ. A 38% decrease in ATP/ADP ratio was observed in euthyroid rats treated with triiodothyronine (109). After elimination of hypothesis 3, Edelman and co-workers looked for evidence of the fourth one. Kinetic studies on rat skeletal muscle showed that triiodothyronine had no effect on K_m for Na^+, K^+ and ATP but increased V_m (6). This result implies that the number of pump sites is increased. Confirmation of this statement was given by studies on rat heart (187). Another approach to show the increased number of ATPase units elicited by thyroid hormones was to incubate rat renal plasma membrane fraction with γ^{32}P-ATP, Mg^{++} and Na^+ (153). The amount of phosphorylated membrane protein was found to increase with increasing Na,K-ATPase activity. Moreover, there was a linear correlation between the specific activity of Na,K-ATPase and the amount of [^{3}H]-ouabain bound to the preparation. However, these results did not distinguish between unmasking of existing units and synthesis of new units by thyroid hormones. To answer this question, Lo and Edelman studied the incorporation of radioactive methionine in the large subunit of renal cortical Na,K-ATPase in response to triidothyronine treatment of rats.

The hormone was reported to increase the incorporation of labeled methionine into the large subunit by 44% 8 hours after infusion of the amino acid and by 61% 20 hours after infusion.

Edelman suggests that triidothyronine-induced synthesis of Na,K-ATPase units is initiated at the level of the genome: a hormone-receptor complex would bind to the chromatin of target-cell nuclei and promote synthesis of specific classes of RNA, including mRNA. Translocation would occur and result in an increased number of Na,K-ATPase units (52).

Although this hypothesis has been accepted by other authors (110,151,241), a triiodothyronine-dependent-increased [^{3}H]ouabain binding, not due to an increased number of binding sites but rather to a change in affinity, has been reported recently in rat heart and skeletal muscle (209). Criticisms of this hypothesis have been reviewed by Himms-Hagen (89). There are several assumptions underlying Edelman's hypothesis. One of them is that changes in intracellular ion composition due to inhibition of the sodium pump do not modify cellular respiration. However, inhibition of the Na,K-ATPase by ouabain causes K^+-depletion. K^+ is required for controlled mitochondrial respiration (see 89). Thus, it is possible that inhibition of oxygen consumption is secondary to K^+-depletion rather than inhibition of Na,K-ATPase. A second assumption is that ouabain and lack of Na^+ block the sodium pump specifically. However, ouabain affects other parameters. For example, ouabain inhibits white adipose tissue adenylate cyclase, sti-

mulates gluconeogenesis in kidney, inhibits gluconeogenesis in liver, promotes glucose uptake in white adipose tissue and muscle (89). Thus, the action of ouabain is not really specific, particularly when used on whole tissues. Moreover, the effects of ouabain are variable. Ouabain has little influence on respiration of normal liver (110); it even stimulates the oxygen consumption in cerebral cortex of guinea pigs (119). This lack of inhibition of respiration by ouabain is apparently due to the presence of Ca^{++} in the incubation medium. A tissue incubated in absence of Ca^{++} rapidly loses its K^+ (89); inhibition of the Na,K-ATPase by ouabain prevents K^+-re-entry and thus causes inhibition of respiration indirectly. When Ca^{++} is present however, the tissue does not readily lose its K^+ and inhibition of the Na,K-ATPase by ouabain appears to have little influence on the respiration. A third assumption is that the phosphorylation state ratio still controls the mitochondrial respiration in the hyper/hypothyroid state and finally that changes in Na,K-ATPase activity measured in vitro reflect altered activity in vivo as well as altered tissue respiration.

In summary, thyroid hormones appear to increase the number of sodium pumps in some tissues (liver, kidney, heart, skeletal muscle). Because of many assumptions made, this mechanism is not however recognized as the unique explanation of the thyroid hormone-induced increase in metabolic rate.

A(iii). Hormonal regulation of Na,K-ATPase.

Apart from regulation by thyroid hormones, Na,K-ATPase appears to be regulated by a number of compounds and other

hormones, the effects of which are variable, depending on the tissues and species.

Na,K-ATPase is subject to inhibition by Ca^{++} (46), prostaglandins (120), acetylcholine (206), palmitoylcarnitine and palmitoyl CoA (160) and serotonin (63). Insulin has been reported to activate Na,K-ATPase (103), although it usually activates the rate of Na^+-K^+ transport only, leaving the enzyme activity unchanged (42,164) or counteracts the inhibitory effect of adrenaline, cyclic AMP and glucagon in rat liver (13,157).

The role of catecholamines in Na,K-ATPase regulation has already been mentioned (section II.2.B.ii). It appears that their mechanism of action is complex. Catecholamines have been shown to stimulate the Na,K-ATPase of a brown adipose tissue membrane fraction through α- as well as β-adrenergic receptors. Cyclic AMP was also effective in stimulating the enzyme (98). In a beef brain microsomal preparation (83) and in rat sarcolemma (37), stimulation did not seem to occur through β-adrenergic receptors nor cyclic AMP. Any orthodihydroxybenzene compounds appeared to increase the Na,K-ATPase activity. However, the stimulatory effect of catecholamines on Na^+-K^+ transport on whole tissue (skeletal muscle) was reported to occur through β-adrenergic receptors and cyclic AMP (41,198). Recent work has demonstrated that the nonspecific stimulation of Na,K-ATPase by catecholamines in various preparations is frequently an artefact due to

reversal by these compounds of inhibition by a contaminant of some commercial preparations of ATP: this is discussed more fully in section IV.4.C.

In summary, the Na,K-ATPase is the enzyme which catalyses the transport of Na^+ and K^+. Na^+ is pumped outside whereas K^+ is pumped inside the cell. This enzyme is confined to plasma membranes and uses ATP as the driving force for transport of ions. The reaction mechanism is complex and involves the formation of a phosphorylated intermediate. Na,K-ATPase is inhibited specifically by cardiotonic steroids (e.g. ouabain) which bind on the outer face of the membrane. Upon purification, two major polypeptides are isolated, corresponding to approximately 100 000 and 55 000. Lipids play an important role in Na,K-ATPase activity. The sodium pump appears to contribute to a large extent to tissue respiration and heat production. The control of respiration by Na,K-ATPase is the basis of Edelman's hypothesis of thyroid thermogenesis. In this hypothesis, thyroid hormones would act by increasing the number of sodium pump sites. The increased ATP hydrolysis would, in turn, stimulate respiration and thus heat production. Na,K-ATPase is subject to regulation by other hormones, in particular catecholamines. Thus, it seems possible that Na,K-ATPase could contribute to the enhanced calorigenic response observed in the cold-acclimated rat after catecholamine administration.

B. β-Adrenergic receptors and adenylate cyclase.

Initially, cyclic AMP was identified by Sutherland and coworkers as the intracellular mediator of the glycogenic effect of adrenaline and glucagon in liver (223). The concept of second messenger was introduced after these studies. Since that time, the role of adenylate cyclase and cyclic AMP in the mediation of various hormonal effects has been studied to a great extent (see 194,224). Although the enzymatic system is usually represented as a simple molecule which catalyses the formation of cyclic AMP from ATP, its structure is much more complex. The recent advances in the knowledge of the enzyme system, especially at the level of the relationship between receptor and enzyme, will be reviewed in some detail. Because we are interested in the action of catecholamines and how their effect is regulated outside and inside the cell, this review will emphasize mainly the control of the adenylate cyclase system at the level of the adrenergic receptor and the enzyme itself.

B(i). Various types of adrenergic receptors.

In 1948, Ahlquist (2) demonstrated the existence of two main types of adrenergic effects: α and β. The relative orders of potency of a series of catecholamines for stimulation of a variety of physiological processes served as a basis for this concept. α-Receptor-mediated responses, typified by vasoconstriction, are very sensitive to agonists

such as adrenaline and noradrenaline but not to isoproterenol, a synthetic amine. β-Receptor-mediated responses, such as cardiac stimulation or bronchodilatation, are stimulated by isoproterenol with noradrenaline much less potent. Each effect is antagonized by specific compounds. Phentolamine and phenoxybenzamine for example are α-adrenergic antagonists whereas propranolol and alprenolol are of the β-type. β-Adrenergic receptors are not identical in all tissues. For this reason, they have been further subdivided into β_1- and β_2-subtypes (133). β_1-Receptors are found, for example, in adipose tissue and cardiac tissue and mediate respectively the lipolytic effect of catecholamines and the cardiac inotropy. β_1-Receptors have an affinity for isoproterenol five to ten times higher than for noradrenaline. On the other hand, β_2-receptors mediate the metabolic effect of catecholamines in muscle and liver. They are also found in vascular, bronchial and uterine smooth muscle. Noradrenaline is less potent than adrenaline which is in turn 1/100 to 1/1000 as potent as isoproterenol.

A third type of adrenergic receptor has been defined more recently (111). It is stimulated to a great extent by dopamine, a precursor of noradrenaline. These receptors play a role in renal vasodilatation and central neurotransmission and are referred to as dopamine receptors.

The structure of some agonists and antagonists is given in Fig. 13. β-Adrenergic receptors have been studied

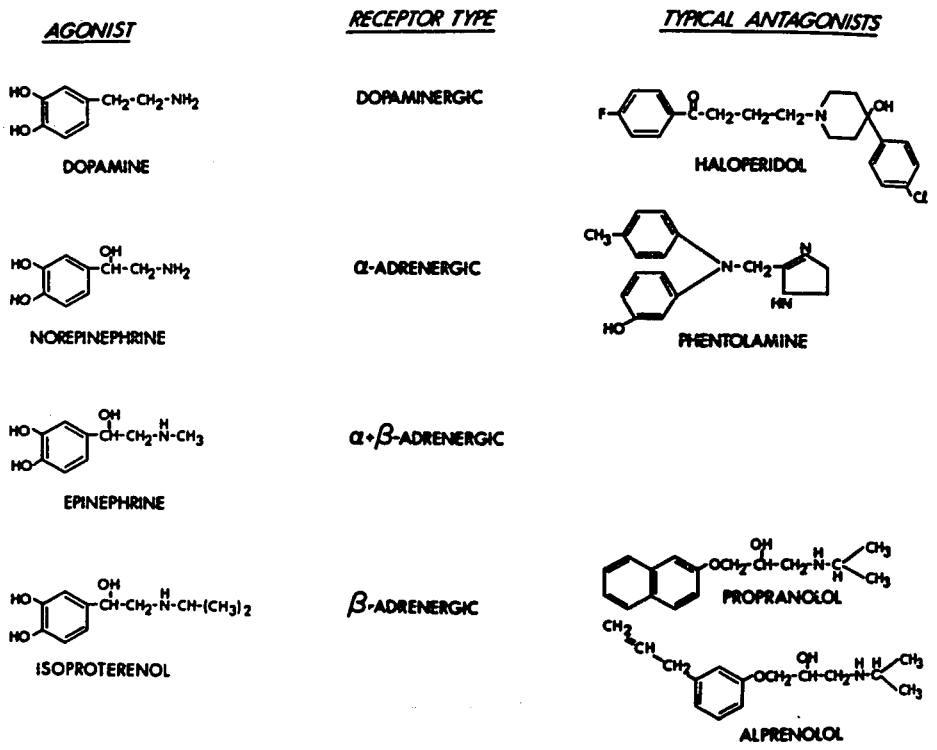

Fig. 13. Structures of some adrenergic agonists and antagonists.

[From Lefkowitz (141)].

in more detail than α- and dopaminergic receptors (see 77, 137,139,141,143). Binding of an agonist to a β-adrenergic receptor mediates a biologic response. The link between the binding and the response is, as mentioned above, the second messenger. In almost all tissues containing β-adrenergic receptors, stimulation of adenylate cyclase by catecholamines occurs and leads to the synthesis of cyclic AMP (193).

B(ii). Identification of β-adrenergic receptors.

The traditional pharmacologic approach to the study of receptor characteristics is based on observation and quantification of physiological responses elicited by agonists and antagonists. Although much information has been obtained through this approach, it is the use of direct radioligand-binding methods in the last few years which has led to major findings in the identification of β-adrenergic receptors.

A major concern is the question of criteria for β-adrenergic receptor identification. These criteria are related to (a) the kinetics, (b) the specificity and (c) the affinity of binding of the labeled adrenergic ligand.

(a) Stimulation of adenylate cyclase by catecholamines and inhibition by antagonists have been shown to occur within a few minutes (171). The labeled adrenergic ligand has to show the same pattern of rapidity and reversibility of binding.

(b) Specificity of binding is assessed by the relation-

ship between the ability of adrenergic agents to compete with the labeled ligand and their pharmacological potency.

(c) Affinity of an agent for a receptor is a measure of the potency in eliciting or blocking a specific biological effect. Equilibrium dissociation constants (K_D) of drugs measured by indirect pharmacologic studies and direct binding studies should fall in the same range (62).

Criteria for β-adrenergic receptor identification have been reviewed in detail by Lefkowitz and coworkers (139,143).

Early studies with radiolabeled catecholamines and antagonists did not give the expected results, the catechol moiety being apparently responsible for a high nonspecific binding (77). Compounds such as [^{3}H]-isoproterenol or [^{3}H]- and [^{14}C]-propranolol appeared not to follow the criteria outlined above (137,143). Recently, identification of β-adrenergic receptors has been achieved by use of (-)[^{3}H]-dihydroalprenolol ((-)[^{3}H]DHA). This compound is obtained after catalytic reduction of the double bond on the aliphatic side chain of alprenolol in presence of ^{3}H gas (see Fig. 13 for structure). (-)[^{3}H]DHA has a very high affinity for adenylate cyclase-coupled β-adrenergic receptors. K_D values for the receptor (0.015 μM) are several orders of magnitude lower than those of potent agonists such as isoproterenol (1 μM) (172). The specificity of (-)[^{3}H]DHA for β-adrenergic receptors is

enhanced by the absence of a catechol group. Moreover, (-)-[^{3}H]-dihydroalprenolol with a relatively high specific activity can be prepared. Due to the small number of β-adrenergic receptors present on the membrane of a single cell, high specific activities are needed to detect any binding of the ligand. For the same reason, it is recommended to work with isolated membrane preparations rather than homogenates in order to achieve high enough receptor concentrations. (-)[^{3}H] DHA appears to meet the criteria outlined above (140).

A number of studies have been done with radiolabeled β-adrenergic antagonists, especially (-)[^{3}H]dihydroalprenolol. These include work with amphibian erythrocytes (140,144,149, 171,173), avian erythrocytes (24) as well as mammalian tissues such as human lymphocytes (237), heart (4) and pineal gland membranes (242).

More recently, the early problem of specificity encountered with labeled catecholamines has been circumvented by use of antioxidants such as ascorbic acid and compounds such as catechol which suppress nonspecific binding. Thus, Lefkowitz & Williams reported a very specific binding of the potent catecholamine agonist (±)[^{3}H]-hydroxybenzylisoproterenol on frog erythrocytes (145). The advantage of working with agonists rather than antagonists resides in the fact that not only binding can be studied but also adenylate cyclase stimulation.

B(iii). Control of the number of β-adrenergic receptors.

The availability of these new techniques permits the direct study of the molecular basis of physiological regulation of β-adrenergic receptors. Chronically elevated levels of hormones or drugs lead to reduced sensitivity of the tissue exposed to that pharmacological agent. Injection of frogs with β-adrenergic catecholamine (isoproterenol) for 1 to 24 hours, led to a marked decrease in the sensitivity of erythrocyte membrane adenylate cyclase to isoproterenol stimulation in vitro (172); basal and fluoride-stimulated activity were unaffected, however there was a 60% fall in the number of β-adrenergic receptor binding sites. These changes in catecholamine-sensitive adenylate cyclase and alprenolol binding are not due to occupancy of β-adrenergic receptors in the membranes by previously injected catecholamines: basal enzyme activity was not elevated; the affinity of the binding sites appeared unaltered. Similar results have been obtained in vitro: erythrocytes were incubated with isoproterenol and membranes isolated. Again, a 63% decrease in isoproterenol stimulated adenylate cyclase activity was observed (166). Stimulation of adenylate cyclase by prostaglandin E_1 was unaffected by isoproterenol preincubation of the erythrocytes, showing that the β-adrenergic system was specifically desensitized (166). In a next step, the same group used a cell-free system: purified frog erythrocyte membranes. Here again,

they demonstrated this desensitization phenomenon. In addition, the specificity of desensitization has been studied on this cell-free system (174). A variety of β-adrenergic agents was tested for ability to desensitize the β-adrenergic receptor binding sites. As shown in Fig. 14, desensitization requires occupancy of the β-adrenergic receptor. Thus the (+) isomer of isoproterenol, which has only about 1/500th the affinity of (-) isoproterenol for the receptors, did not desensitize the binding sites. Noradrenaline which has much less affinity than isoproterenol had only a weak desensitizing effect.

A comparable desensitization phenomenon has been reported in the rat pineal gland, a mammalian system (125). The physiological stimulation of β-adrenergic receptors in the pineal during the night is greater than during the day. The authors reported more hormone-sensitive adenylate cyclase and more (-)[^{3}H]DHA binding in pineals from rats exposed to light 24 hours than in rats kept in the dark for the same time. A decrease in both parameters was observed when the rats exposed to constant light were treated with isoproterenol (125).

If desensitization of the receptors occurs in presence of catecholamine agonists, one can wonder whether treatment of tissues with antagonists or removal of catecholamines would cause an increased sensitivity. It was precisely what happened when rats were treated with the β-adrenergic anta-

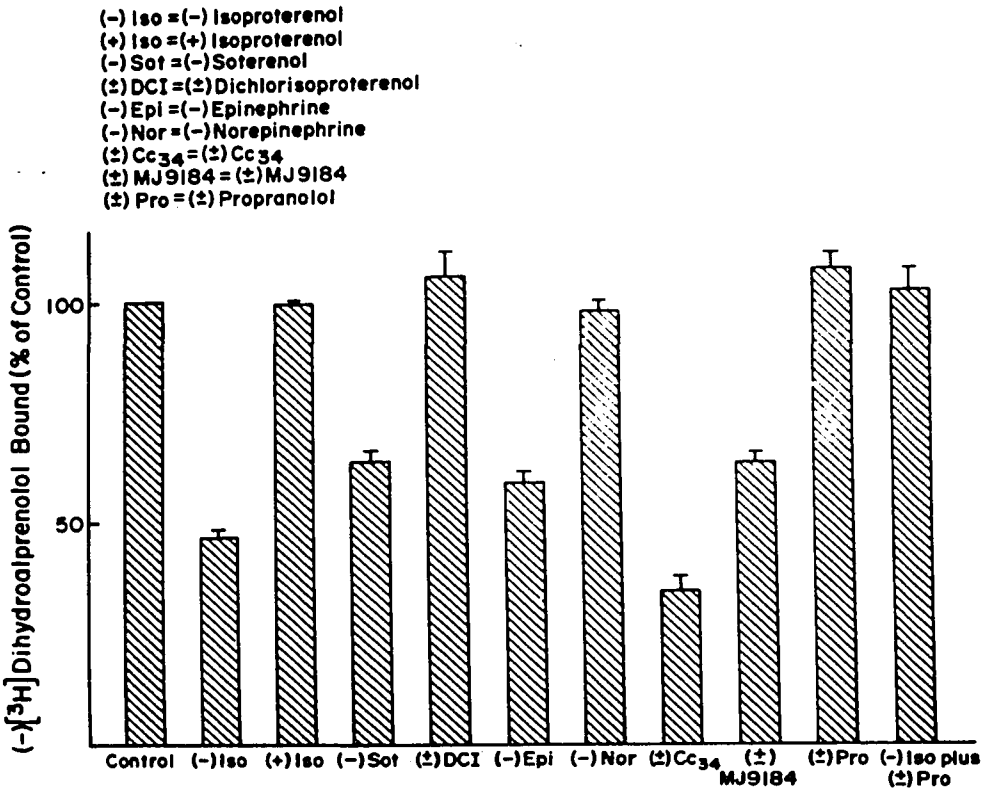

Fig. 14. Effect of preincubation of frog erythrocyte membranes with various β-adrenergic agents on the number of (-)[^{3}H]dihydroalprenolol binding sites.

Preincubations were for 1 hr at 25°C, following which membranes were washed. Control (-)[^{3}H]dihydroalprenolol binding was 1.54 pmoles/mg protein. Values are means ± SEM of duplicate determinations from three experiments. [From Mukherjee & Lefkowitz (174)].

gonist propranolol for two weeks. A 100% increase in the number of β-adrenergic receptors of ventricular membranes was observed (67). An increase in β-adrenergic receptor number in rat brain has also been reported after 6-hydroxydopamine treatment which caused a chronic depletion of tissue noradrenaline (217).

Although the molecular mechanisms are not known yet, tolerance, tachyphylaxis or selective decrease in sensitivity to chronically administered drugs or hormones is now well established. The interesting aspect of the catecholamine-responsive system is that a measurable biologic effect, namely cyclic AMP production can be related to observed changes in receptor binding.

B(iv). Regulation of the adenylate cyclase system by guanyl nucleotides.

In the study on a cell free system reported above (section B.iii.), a discrepancy exists between the extent of β-adrenergic receptor binding site inactivation (60%) and the corresponding adenylate cyclase desensitization (37%) (174). Since the only difference between the adenylate cyclase assays and the binding assays is the presence of ATP, cyclic AMP and an ATP regenerating system in the adenylate cyclase assays, the authors speculated that these reagents might in some way be able to resensitize the β-adrenergic

receptors. ATP and other purine nucleotides were added to desensitized membranes. An increase in the number of β-adrenergic receptor binding sites was observed. Cyclic AMP was without effect, as was the regenerating system alone. 5'-Guanylyl imidodiphosphate (GppNHp) was the most potent in resensitizing the membrane, followed by GTP.

A role of guanyl nucleotides on the activity of the hormone-sensitive adenylate cyclase has been first reported by Rodbell and coworkers for the glucagon-sensitive adenylate cyclase in plasma membranes of rat liver (195,196). Fig. 15 shows a typical response of the adenylate cyclase system to guanyl nucleotides in absence and presence of hormone. In this example (204), isoproterenol and GppNHp activate adenylate cyclase in a synergistic manner, which means to a level much higher than the sum of the activities produced by isoproterenol and GppNHp separately. The authors propose that the function of the hormone in activation of adenylate cyclase is to facilitate the action of the guanyl nucleotide. The effect of GppNHp alone or in combination with isoproterenol is not a simple reversible activation of the enzyme; it is more likely that preincubation with the nucleotide alone or with the hormone affects the transition to a stable activated state. This state is produced by GppNHp but not by GTP probably because the terminal phosphate is cleaved more readily in the case of GTP (204).

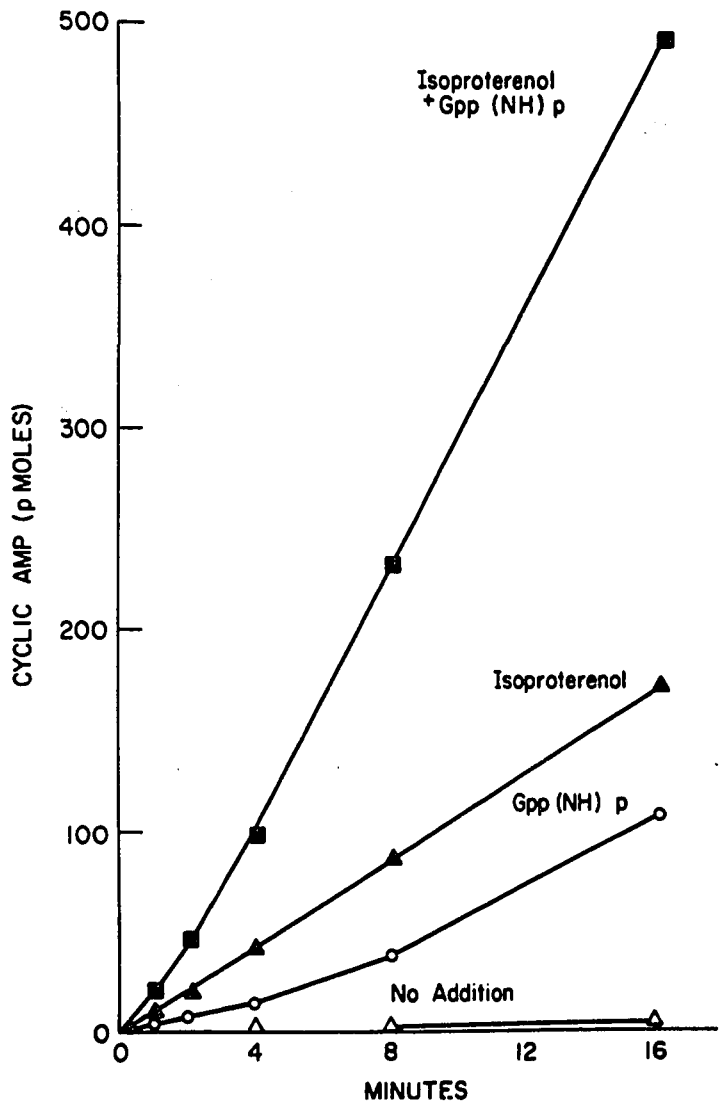

Fig. 15. Activation of frog erythrocyte adenylate cyclase system by isoproterenol, by GppNHp and by isoproterenol plus GppNHp.

[From Schramm & Rodbell (204)].

To explain the characteristics of the system, the following model is proposed (see Fig. 16) (204): the enzyme (E_o) which includes the hormone receptor, the active site and the guanyl nucleotide site, is converted by GppNHp (N) to E_1. E_1 might be the active state of the enzyme induced by GppNHp in absence of hormone; however, it is possible that E_1 is converted further partially to E_3 even in absence of hormone (dotted arrows), E_3 being the highly active state of the enzyme. When both GppNHp and hormone (H) are present, E_o is converted via E_2 to E_3, E_2 being the hormone activated state (204). In addition, conformational alteration of the adenylate cyclase component due to guanyl nucleotides would would in turn modulate conformational alterations of the receptor coupled to adenylate cyclase and thus lead to resensitization of the receptor (174).

B(v). Coupling of receptor to adenylate cyclase: role of lipids.

One important point in the study of the control of adenylate cyclase is the nature of the relationship between the enzyme and the receptor. Several possibilities have been considered and are summarized in Fig. 17, (73).

Although the mechanism of coupling is not known yet, it appears however that at least two different molecules are involved in the system. This statement arises from a work by

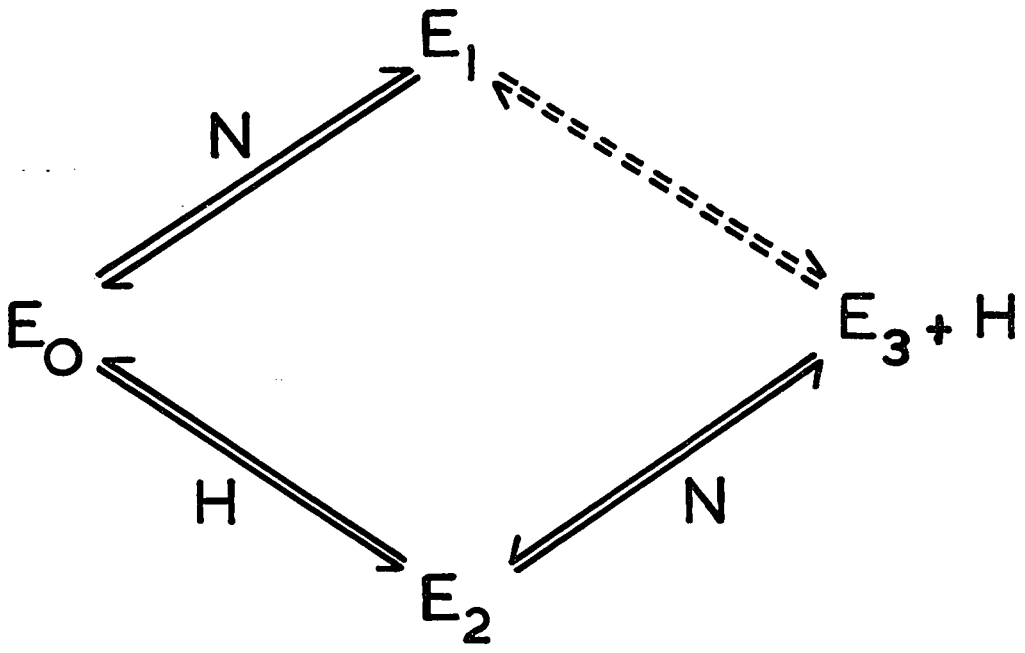

Fig. 16. Activation of the adenylate cyclase system by hormone and nucleotide.

(For explanations see section II.3.B.iv).

Abbreviations: E_0 : adenylate cyclase, basal activity.

E_1 : nucleotide-activated state.

E_2 : hormone-activated state.

E_3 : highly activated state.

H : hormone (isoproterenol).

N : Nucleotide (GppNHp).

[From Schramm & Rodbell (204)].

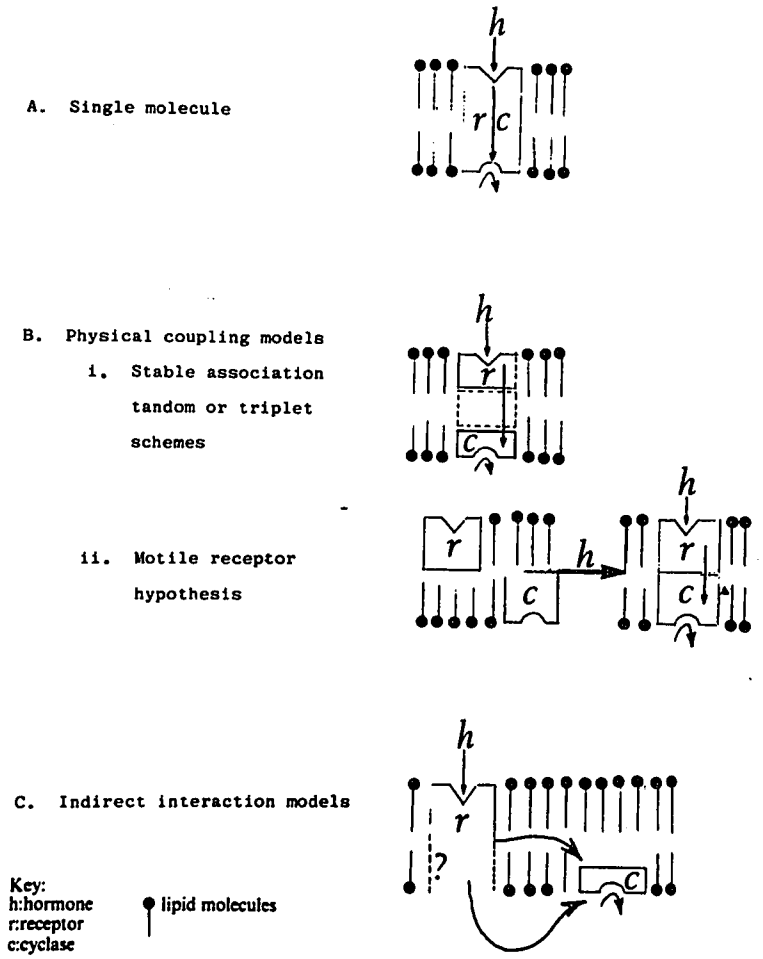

Fig. 17. Models of possible physical relationships between hormone receptors and adenylate cyclase.

[From Greaves (73)].

Orly & Schramm (181). They managed to couple catecholamine receptors from one cell with adenylate cyclase from another cell by cell fusion. The β-adrenergic receptors were taken from turkey erythrocytes in which the adenylate cyclase had been inactivated by N-ethylmaleimide or heat. Friend erythroleukemia cells (F cells) which do not contain measurable β-adrenergic receptors contributed the adenylate cyclase. Fusion was accomplished by Sendai virus. Activation by isoproterenol was demonstrable within a few minutes after fusion, indicating the existence of a coupling between β-adrenergic receptors and adenylate cyclase (181). Further evidence for the autonomy of the receptor and the enzyme is given by Charness and coworkers who demonstrated an independent variation of β-adrenergic receptor binding and catecholamine-stimulated adenylate cyclase activity in rat erythrocytes (35).

Separation of β-adrenergic receptors from adenylate cyclase was obtained in cat ventricular myocardium (142). Independent partition during gel exclusion chromatography was observed for binding and cyclase activities solubilized from erythrocyte membrane with digitonin (150). Molecular weight for the receptor was 130 000 - 150 000 (32). Atlas and Levitski reported the identification of β-adrenergic receptor subunits from rat skeletal myoblasts grown in culture (L6P cells) and from turkey erythrocytes. They iden-

tified two bands, by gel electrophoresis, in the range 37 000 - 41 000 indicating that the receptor might be oligomeric in structure (7). Disparity in molecular weights of cardiac and liver adrenergic receptors (although around 150 000) was reported by use of similar techniques (61).

Thus, numerous lines of evidence indicate that receptor and enzyme are two different entities.

After separation of β-adrenergic receptors from adenylate cyclase by a detergent, the need for phosphatidylinositol (PI) in the activation of adenylate cyclase by noradrenaline was reported (142). The role of lipids in the coupling of β-adrenergic receptors to adenylate cyclase has been assessed by use of filipin (149). This compound forms with membrane cholesterol a filipin-cholesterol complex. Filipin was reported to reduce the isoproterenol-stimulated adenylate cyclase activity without decreasing receptors binding. It was concluded that the alterations produced by filipin are distal to the β-adrenergic receptors and involve the coupling between binding and cyclase activity. Are lipids present only to maintain the membrane architecture or are they responsible for the transmission of the signal from receptor to enzyme? As for Na,K-ATPase, the results are controversial. In a preparation of solubilized cat myocardial adenylate cyclase, unresponsive to histamine, noradrenaline, glucagon and thyroxine after treatment with Lubrol PX, only the histamine and glucagon responsiveness was restored by phosphatidylserine (PS) (147). Specificity was

also observed with PI which restored only catecholamine responsiveness in the same preparation (146). In these two examples however, no binding studies were done. Hence, this specificity could apply to restoration of catecholamine binding rather than coupling. In contrast, less specificity was reported by Lefkowitz in canine cardiac membranes treated with phospholipases A, C or D. Restoration was only achieved by total lipids extracted from the membrane, not by pure phospholipids (138).

B(vi). Summary and conclusions.

Three types of adrenergic receptor have been defined, namely α-, β- and dopaminergic receptors. The physiological β-adrenergic effects of catecholamines occur through stimulation of adenylate cyclase. In the last few years, direct radio-ligand binding studies have permitted to overcome the limits imposed by traditional pharmacologic approach. These studies include the use of high affinity β-adrenergic antagonists (and more recently agonists) to localize β-adrenergic receptors in adenylate cyclase containing membrane preparations. Catecholamines appear to regulate their own receptors: continuous exposure of tissues to catecholamines leads to refractoriness of the tissue to the pharmacological effect of the hormone. This phenomenon called tachyphylaxis or tolerance seems to be regulated not only by the hormone itself but

also by guanyl nucleotides which have the power to resensitize the receptors and also act upon the enzyme adenylate cyclase by activating it. Combination of guanyl nucleotides and catecholamines stimulate adenylate cyclase in a synergistic manner in most cases. Interrelationship between receptor and adenylate cyclase is not very well defined. Recent studies however indicate that there are two different entities and that lipids play an important role in the coupling of the receptor to the enzyme.

Several aspects remain unclear and will probably be worked out in detail in the next few years, namely:

- What are the mechanisms which lead to desensitization of the receptors?
- How are the receptors coupled to the enzyme and how is this coupling regulated?
- What are the physiological implications of the stimulation of adenylate cyclase by guanyl nucleotides and what is the molecular mechanism of this stimulation?

PART 4: TECHNIQUES FOR ISOLATION AND CHARACTERIZATION OF SARCOLEMMA.

The possibility of emptying muscle fibres was reported, for the first time, in 1947 by Barer who treated muscle fibres with dilute acids and observed herniation of muscle content through ruptures of the sarcolemma (12). Three years later, Casella observed empty sarcolemmal tubules after compressing frog muscle fibres with a glass rod in order to study the elastic properties of the empty sarcolemma (33). Sarcolemma has been obtained by boiling bovine muscle fibres and homogenizing them in a Waring blender (231). In 1961, isolation of sarcolemma was accomplished by use of homogenization followed by NaOH and tartaric acid incubation (155). Since that time, numerous techniques for isolation of sarcolemma have been described. In this part, these various techniques will be reviewed as well as the criteria which allow sarcolemma identification.

A. Isolation techniques.

The isolation techniques can be divided broadly into three main types. In the first type, strong salt solutions are used to extract the content of the disrupted cells. These techniques are modifications of the method used originally by Kono and Colowick (130). The second class of isolation procedures in which milder conditions

(low ionic strength) are used, derive from methods designed by Rosenthal et al., (199) and McCollester (158). In the third class are gathered methods which consist of breaking sarcolemma into small vesicles which are further isolated on density gradient. This technique was first used by Kidwai et al., (127). The list of the various techniques as well as the species from which the sarcolemma has been isolated is given in Table I.

A(i). Type (i).

The first step in nearly all methods is to place the muscle pieces after they have been excised from the animal into ice-cold $CaCl_2$ claimed by McCollester to prevent irreversible contraction of the muscle segments during homogenization (158). Then, muscle pieces are cleaned of fat and connective tissue. The second step is the homogenization which can be done with various types of homogenizers such as Virtis Omnimixer (130), Waring blender (1,222), Sorvall Omnimixer (207), Potter-Elvehjem (21,22), or Polytron (5, 175, 203). The homogenization is critical. Short time homogenization leaves the cell fragments too long to permit effective extraction of the cell content. On the other hand, membrane fragments obtained after prolonged homogenization are too small to be recognized as sarcolemma (130). After filtration through cheesecloth or a nylon sieve (to remove connective tissue and large fragments), the suspension is centrifuged and the pellet resuspended in a buffer. This

Table I. Techniques for isolation and characterization of skeletal muscle plasma membranes.

Type (i) (130) (strong salt solutions)	Type (ii) (158,199) (low ionic strength)	(Type (iii) (127) (sucrose density gradient)
bullfrog (1,129)	mouse (47)	frog (21)
frog (21)	rabbit (102)	rat (10,180)
hamster (222)	rat (18,185,229)	
mouse (22)		
rabbit (207)		
	rabbit (163)	
rat (5,175,203)		

This table indicates the origin of the material isolated with the three different types of techniques. References to literature are also given.

procedure allows elimination of soluble enzymes and myofibrils present outside the muscle fibre pieces. The washed segments are then resuspended in 0.4 - 0.5 M LiBr and their contents extracted by stirring them magnetically for several hours at pH > 8, otherwise extraction rate is too slow; on the other hand, too high a pH causes destruction of membranes (130). The resulting solution is viscous and consists of muscle fibre fragments partially freed of their actomyosin content. Then, after centrifugation, the tubular membranes are further extracted by use of KBr or KCl and finally resuspended in distilled water or buffer. Additional steps are reported, consisting in differential centrifugationsin KCl (130) or density gradient centrifugations (22,203).

A(ii). Type (ii).

The first steps (i.e. muscle excision, homogenization, filtration and washing) in these techniques are similar to the ones described for type (i). The extraction is then carried out under mild conditions: incubation at 37°C in buffer or distilled water for thirty minutes, followed by washings and extraction by distilled water (158), buffer (47) or diluted solutions of NaOH (199) and eventually by solutions of ATP (185,199). ATP is supposed to act on the actomyosin in dissociating the molecule into its constituent proteins: myosin and F-actin. This leads to a rapid reduction in the viscosity of the solution.

A(iii). Type (iii).

For this type, the approach is different: after vigorous homogenization and filtration, the suspension is loaded on the top of a sucrose gradient. After centrifugation, the fraction corresponding to plasma membrane is collected and washed. By use of this technique, the extraction procedures described above are eliminated but, because of the small size of the particles, possibilities of contamination by other subcellular membrane fractions might occur.

B. Characterization of sarcolemma.

Sarcolemma is usually characterized in three ways: morphology, enzymatic activity and chemical composition.

B(i). Morphology.

The light microscope or phase contrast microscope are used to define the morphology of isolated sarcolemma. After the final step, it has the appearance of empty tubes (130, 158,199,229) or sheets (47). When sarcolemma is isolated on density gradient, electron micrographs show vesicles of various sizes (127,180). Electron micrographs also permit the description of the structure of the membrane. According to Rosenthal et al., (199) four components can be seen:

- on the outside of cell, a mesh of fine filaments 300 Å in width,
- then, collagen filaments 300 Å in width;
- the next layer is an amorphous zone and measures 300 -

500 Å in width;

- finally, the plasma membrane itself (100 Å), adjacent to the cytoplasm.

This structure represents the true sarcolemma; only the plasma membrane is present in vesicles (47). Type (i) and (ii) techniques allow isolation of true sarcolemma whereas type (iii) techniques permit isolation of plasma membrane fragments.

B(ii). Enzymatic markers.

Enzymatic markers are used to assess the purity of the sarcolemma as well as to appreciate the contamination by other components of the cell such as mitochondria, sarcoplasmic reticulum or lysosomes.

Na,K-ATPase and 5'-nucleotidase have been recognized as plasma membrane markers. Values in the range between 2 and 31 µmoles/mg protein.hour have been reported for Na,K-ATPase activity in different species and preparations (5,10,22,47,127,175,185,203,207,222). 5'-Nucleotidase activities vary from 1.65 to 11.7 µmoles/mg.protein.hour (127,180,203). Acetylcholinesterase has also been used as a sarcolemma marker (22,47,207).

Other enzyme assays are currently done to check for any contamination; for example cytochrome oxidase (mitochondria inner membrane) (22,127,207), acid phosphatase (lysosomes) (47,203,207), lactate dehydrogenase (cytoplasm) (47, 203). Usually sarcoplasmic reticulum contamination is not assessed, no suitable marker enzyme being available (47).

B(iii). Chemical composition.

Little information is available for the protein and carbohydrate composition of sarcolemma. Amino acid analysis shows a high content of acidic amino acids (Asp & Glu), glycine, proline and hydroxyproline in frog (1) and rat (130, 229) sarcolemma. Collagen seems to be a relatively minor and variable constituent of isolated sarcolemma (1,162,229). The collagen filament layer observed by Rosenthal et al., (199) and Kono et al., (131) is probably not part of the membrane; this layer can be removed by washing with hypertonic KCl during the extraction. Vandenburgh et al., (229) reported three major bands on SDS-gel electrophoresis, corresponding to proteins of 170 000, 140 000 and 44 000. Polypeptides of 110 000 and 140 000 were observed by Andrew & Appel (5) as well as a double peak around 93 000 which could correspond to Ca, Mg-ATPase. A total of twenty polypeptides was reported by these authors. The pattern obtained by Madeira and Antunes-Madeira (162) is quite different and appears to show contamination by collagen.

Sialic acid has been used as a marker for plasma membranes by several authors (22,47,207). In the study by Andrew & Appel (5) on rat sarcolemma, sialic acid concentrations for membrane fractions were 25 to 50 times greater than for the whole muscle. Moreover, the distribution of

sialic acid on the continuous density gradient paralleled the Na,K-ATPase activity.

A general characteristic of plasma membranes appears to be a higher cholesterol content compared to that of intracellular membranes (127). The absolute values for sarcolemma vary from 3.72 µg/mg protein (47) to 264 µg/mg protein (53). The cholesterol to phospholipid ratio is also higher in plasma membranes (0.5 to 1.3 depending on the tissue) (43). For sarcolemma, a ratio of 0.37 was reported by De Kretser and Livett (47). Although a ratio of 0.11 was found by Kidwai et al., (127), it is still higher than those calculated for the other fractions isolated together with sarcolemma (sarcoplasmic reticulum : 0.04; mitochondria : 0.025). Among phospholipids, usually a high percentage of lecithin is reported (25 to 67%) (1, 47, 162). Fatty acid analysis of the phospholipid fraction indicates a high proportion of saturated fatty acids in sarcolemma compared to sarcoplasmic reticulum and mitochondria (53). High cholesterol and saturated fatty acid concentrations have been suggested to contribute to tighter packing of phospholipids in the bilayer (161). This explains the increased stability and impermeability of sarcolemma by comparison with other membranes.

CHAPTER III: MATERIALS AND METHODS

PART 1 : MATERIALS.

A. Rats.

Male Charles River Holtzman rats of the same age, weighing 100-125 grams, were purchased in groups from Canadian Breeding Laboratories (Lasalle, Quebec). After one week in group cages (about 12 rats per cage) at room temperature (27 ± 1°C), they were randomly transferred to individual wire cages and divided into two groups: one group served as control (warm-acclimated) rats and was maintained at room temperature (27 ± 1°C), whereas the second group was placed in a cold room (4 ± 1°C) at least four weeks before use, to ensure that the rats were fully acclimated to cold. Rats had free access to water and food, and artificial lighting was maintained in both rooms 12 hours a day (6-18 hrs). Rats were weighed once a week and before each experiment; only rats showing a normal development were used. The experiments were designed so that animals were studied in pairs, the only variable being the acclimation temperature. The order in which they were killed was alternated from one experiment to the next. This design was used to reduce variability between animals due to age, sex, duration of exposure to cold, age at initial exposure to cold, diet and handling, and to reduce variability in experimental procedures due to equipment, solutions, manipulation and duration of preparative techniques.

B. Chemicals.

The following chemicals were purchased from the Sigma Chemical Co.:

Albumin (bovine fraction V), cAMP (adenosine 3',5'-cyclic monophosphoric acid), ATP (adenosine 5'-triphosphate, diso-

dium salt), ATP (vanadium-free) (adenosine 5'-triphosphate, disodium salt), tris-ATP (tris(hydroxymethyl)-aminomethane ATP), 5'-AMP (adenosine 5'-monophosphoric acid), alumina (chromatographic neutral, type WN-3), noradrenaline (L-arterenol bitartrate), ascorbic acid (sodium salt), calcium chloride, choline chloride, Coomassie blue R250, cytochrome c (horse heart, type III), EDTA (ethylenediamine tetraacetic acid, disodium salt), EDTA (free acid), EGTA (ethyleneglycol -bis (β-aminoethyl-ether)-N,N'-tetraacetic acid), L-epinephrine bitartrate, GppNHp (5'-guanylyl imidodiphosphate, sodium salt), L-histidine, imidazole, lubrol WX, ouabain (octahydrate (strophanthin-G), PPO (2,5-diphenyloxazole), PMSF (phenylmethylsulfonylfluoride), phospho(enol)pyruvate (monopotassium salt), potasssium bromide, KH_2PO_4 (potassium phosphate monobasic), DL-propranolol-HCl, pyruvate kinase (type III), trizma-HCl (tris[hydroxymethyl]aminomethane hydrochloride), trizma-base (tris[hydroxymethyl]aminomethane), and sucrose.

Lithium bromide, magnesium chloride, potassium bromide, ammonium molybdate, potassium chloride, ferrous sulfate, potassium phosphate (monobasic) were obtained from Fisher Scientific Co.;sodium bisulfite, potassium bicarbonate, sodium fluoride and toluene from J.T. Baker, Chemical Co.; magnesium sulfate and perchloric acid from The British Drug house LTD and acrylamide, ammonium persulfate, bis (N,N'-methylene-bis-acrylamide), Dowex 50 AG WX4, 2-mercaptoethanol, silicic acid (Bio-Sil A, 100-200 mesh), Temed (N,N,N',N'-

tetramethyl-ethylenediamine) from BioRad Laboratories; Silica gel H, type 60 from Brinkmann Instruments; amidol (2,4-diaminophenol dihydrochloride) from Eastman Kodak. Phospholipid standards were purchased from Serdary Research Laboratories, fatty acid standards from Nu Check Prep.

[^{3}H]-cAMP (^{3}H-adenosine 3',5'-cyclic monophosphoric acid), [^{3}H]-alprenolol (dihydroalprenolol hydrochloride, levo-[propyl-2,3-^{3}H]), [^{14}C]-sucrose (^{14}C-U) and Aquasol (liquid scintillation counting cocktail) were obtained from New England Nuclear; [α^{32}P]-ATP from New England Nuclear (adenosine 5'-triphosphate, tetra (triethylammonium) salt [^{32}P]) or Amersham Corporation (Adenosine 5'-[^{32}P]triphosphate, sodium salt); NCS tissue solubilizer and PCS (liquid scintillation counting cocktail) from Amersham Corporation.

PART 2 : METHODS.

A. Isolation of sarcolemma.

A(i). Technique I.

Two different methods have been used to isolate sarcolemma. In the first one, high salt concentrations were used. It will be referred to as technique I. This extraction procedure was adapted mainly from methods designed by Rosenthal et al., (199) and Severson et al., (207) with some contribution from methods described by the following authors: Sulakhe et al., (222), Peter (185), McCollester (158), McCollester & Semente (159) and Sulakhe et al., (220,221).

Shortly before the experiment, rats were taken to the laboratory from their respective temperature-controlled rooms, weighed and killed by decapitation. Hind leg muscles as well as muscles from the back were immediately excised and immersed in ice-cold 50 mM $CaCl_2$. All further procedures were performed at 0 - 4°C. After removal of fat and connective tissue, muscles were minced with scissors and 30 g of the muscle mince were placed in 100 ml of ice-cold 50 mM $CaCl_2$. The suspension was then homogenized at high speed for 10 seconds in a Waring blender with reversed blades (see 199). The homogenate was filtered through a coarse nylon sieve (pore size: 1 mm^2). The residue was rehomogenized in 100 ml of 50 mM $CaCl_2$ and filtered the same way; this operation was repeated two additional times. The pooled homogenate was refiltered and centrifuged at 3000 g for 5 seconds in 50 ml cellulose nitrate tubes in a Sorvall RC2-B centrifuge (SS-34 rotor); the supernatant was discarded. The sediment was resuspended in 150 ml KCl buffer (45 mM KCl, 30 mM $KHCO_3$, 2.5 mM L-histidine pH 7.8 with tris-HCl), homogenized by hand in a loose-fitting Potter-Elvehjem homogenizer and recentrifuged as above. This washing procedure was repeated two additional times. The resulting sediments were combined, resuspended in 150 ml of a LiBr solution (0.5 M LiBr, 0.005 mM EGTA, 16 mM tris, pH 8) and stirred gently overnight (15 hrs) at 4°C. The viscous suspension was then diluted with 200 ml KCl buffer and centrifuged 10 minutes at

1500 g (SS-34 rotor). The resulting pellet was resuspended in 100 ml KCl buffer, homogenized as above and centrifuged 10 minutes at 1475 g. This operation was repeated one additional time. The partially extracted, washed membrane tubes were resuspended and homogenized in 50 ml 25% KBr (w/v), allowed to stand for 10 minutes and centrifuged 30 minutes at 18 500 g (SS 34 rotor). The pellet was resuspended and homogenized (20 passes in the loose-fitting homogenizer by hand) in 80 ml of 0.4 mM ATP adjusted to pH 7.4 with tris-base. The suspension was centrifuged 5 minutes at 3000 g. This last operation was repeated four additional times and allowed the extraction of the actomyosin trapped in the membrane tubes (see 199). The pellet was then resuspended and homogenized in 80 ml distilled water pH 7.4 (adjusted with tris-base) and centrifuged 5 minutes at 18 500 g (SS 34 rotor). The final pellet constituted the sarcolemma preparation. It was resuspended in distilled water pH 7.4 and homogenized for one minute in a Potter-Elvehjem homogenizer with a teflon pestle at 5000 rpm before use.

A(ii). Technique II.

The second technique will be referred to as technique II and differed from technique I in that no salt was used but rather plasma membranes were isolated at the surface of a 29% sucrose solution after high speed centrifugation. This

technique II was adapted from the method described by Kidwai et al., (127).

Muscles were placed in ice-cold 0.25 M sucrose, cleaned of fat and connective tissue and cut into small pieces with scissors. All subsequent steps were carried out in the cold room (0 - 4°C). 5 g - batches in 30 ml 0.25 M sucrose were homogenized for 10 seconds with a Polytron PT 20 (Brinkmann Instruments) at a setting of 8. Homogenization was done in glass tubes with a diameter slightly greater than that of generator PT 20. The glass tube stood in an ice bath. The suspension was then filtered through a filtration device similar to the one designed by Kidwai (126). The filtrate was centrifuged at 32 000 rpm (about 100 000 g) in a Beckman L2-65B ultracentrifuge (60 Ti rotor) for 30 minutes. The pellet was resuspended in a minimal volume of 0.25 M sucrose, loaded on a 29% (w/v) sucrose solution contained in polyallomer tubes and centrifuged for two hours in a SW 27 rotor at 24 000 rpm (about 112 000 g). [Kidwai et al., (127) used a sucrose density gradient to isolate various subcellular membrane fractions. The use of one sucrose solution constitutes the major modification of the method described by Kidwai et al.] At the end of the run, the zone at the interface of the loading suspension and the sucrose solution was collected with a Pasteur pipette, diluted four times with distilled water pH 7.4 (adjusted with tris-base) and recentrifuged at 100 000 g for 30 minutes as

for the first centrifugation. The resulting pellet was resuspended in distilled water pH 7.4 and recentrifuged the same way. The final pellet was resuspended in a small volume of distilled water pH 7.4 (1 - 2 ml).

B. Protein estimation.

Protein content was estimated by the Lowry method (156) as modified by Schacterle & Pollack (202). Since compounds such as sucrose and tris have been found to interfere with the Lowry protein determination (118), samples used for protein estimation were precipitated with ice-cold 12.5% (w/v) trichloracetic acid, let to stand in ice for 30 minutes and centrifuged at 16 500 g for 15 minutes. The pellet was dissolved in 2 ml of 0.5 N NaOH and 1 ml of this solution assayed according to Schacterle and Pollack (202).

C. Microscopy.

Morphological controls and photographs were done by phase-contrast microscopy with a Carl Zeiss photomicroscope.
Magnification : x200
Film type: Panatomic X-135

D. Cytochrome oxidase. (EC 1.9.3.1)

The sarcolemma was homogenized for one minute with lubrol WX (1.5 mg/mg protein). Cytochrome oxidase 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 ml. Cytochrome c solution was prepared by dissolving 1 g of cytochromec in 100 ml of 0.01 M potassium phosphate buffer pH 7.4 and adding 100 mg ascorbic acid (see 233). The concentration of total and reduced cytochrome c was checked using the extinction coefficients reported by Yonetani (240). Activity was expressed as µatoms oxygen consumed/mg protein. min.

E. 5'-Nucleotidase (EC 3.1.3.5)

5'-Nucleotidase was measured as recommended by Solyom & Trams (216). 100 µl membrane suspension (0.2 to 0.6 mg) were incubated with 200 µl "tris buffer" (75 mM tris, 10 mM KCl, 5 mM $MgCl_2$, 5 mM 5'-AMP) pH 9.0 for 15 minutes at 37°C. Reaction was stopped by addition of 1 ml cold 12.5% trichloracetic acid (w/v). Released phosphate was estimated by the method of Taussky & Shorr (226).

F. Na,K-ATPase (EC 3.6.1.3). Mg-ATPase (EC 3.6.1.4)

ATPases were assayed according to Severson et al., (207), Sulakhe et al., (222) and Peter (185). The method consists of measuring phosphate released from ATP in the reaction catalysed by plasma membrane ATPases. Sarcolemma (0.1 - 0.2 mg) was preincubated at 37°C for 3 minutes in a

medium composed of 50 mM tris, 1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl adjusted to pH 7.4. The reaction was started by the addition of 4 mM tris-ATP, Na-ATP or Na-ATP vanadium-free (final volume : 1 ml) and allowed to proceed for 10 minutes. The reaction was stopped by the addition of 1 ml cold 12.5% trichloracetic acid (w/v), and the phosphate released measured as described by Taussky & Shorr (226). Na,K-ATPase activity was assessed from the inhibition by ouabain (1 - 2 mM) or from the inhibition by substitution of choline chloride (120 mM) for NaCl and KCl to maintain the same ionic strength in absence of these ions (see 112). The remaining activity, after inhibition of the Na,K-ATPase, was taken as the Mg-ATPase activity.

G. Adenylate cyclase (EC 4.6.1.1)

Adenylate cyclase was assayed according to Salomon et al., (200). The method consists of measuring the production of labeled cyclic AMP from the substrate [α^{32}P]ATP. The assay system (total volume: 200 µl) was composed of 40 mM tris, 2 mM cAMP, 5.5 mM KCl, 15 mM $MgSO_4$, 1 mM EGTA, 20 mM phospho(enol)pyruvate, 125 µg/ml pyruvate kinase and 100 - 200 µg sarcolemmal protein. NaF, GppNHp, noradrenaline were added to the incubation medium when needed. All solutions had been previously adjusted to pH 8.5. After 3 minutes preincubation at 37°C, 20 µl [α^{32}P]ATP (final concentration : 2 mM, 5 - 10 µCi) was added to start the reaction.

After 15 minutes incubation, the reaction was stopped by addition of 20 µl 0.31 M Na-EDTA pH 7.6 containing [^{3}H]cAMP (0.05 µCi). Each tube was boiled 3 minutes. A control consisted of all the components except sarcolemmal protein. Sarcolemma was added, in this tube, 30 seconds before the end of boiling. Then, the tubes were cooled in iced water and 1 ml distilled water was added to each tube, which brought the volume to 1220 µl. After mixing, the tubes were centrifuged for 15 minutes at 3000 g (SM-24 Sorvall) and decanted into columns (Pasteur pipettes plugged with glass wool) containing 1 ml Dowex 50 AG XW4 resin. The eluate from this addition and from two successive 1 ml H_2O washes were discarded. Three ml of H_2O were added to the Dowex column, allowed to pass directly through an alumina column (Pasteur pipette filled with 0.6 g neutral alumina) and discarded. Four ml 0.1 M imidazole-HCl pH 7.5 were subsequently added to the alumina column and the eluate collected directly in scintillation vials containing 10 ml PCS (a cocktail for liquid scintillation counting of aqueous samples). ^{32}P and ^{3}H were counted in a two channel Beckman liquid scintillation counter (LS-250). Channel discriminators were adjusted so that 1-2% of ^{32}P counts entered the tritium channel without any tritium counts entering the ^{32}P channel. After correction for recovery of ^{3}H cAMP and subtraction of the blank values, results were calculated as pmoles cyclic AMP produced/mg protein.min.

H. (-)[^{3}H]Dihydroalprenolol binding.

This method was adapted from the binding assay described by Alexander et al., (4). The assay system (total volume: 170 µl) consisted of 100 µl sarcolemmal protein (0.1 - 0.5 mg) in "incubation buffer" (75 mM tris, 25 mM $MgCl_2$ pH 8.1), 10 µl [^{14}C]-sucrose (about 100 000 cpm) ± 10 µl GppNHp (0.12 mM). The incubation was started, after 3 minutes preincubation at 37°C, by addition of 25 µl (-)[^{3}H]-dihydroalprenolol (final concentration: 30 nM; 20 Ci/mmole). The incubation time was 10 minutes at 37°C. Nonspecific binding was assessed by incubation in presence of propranolol (final concentration : 100 µM). At the end of the incubation, 100 µl aliquot was pipetted into a 1.5 ml Eppendorf micro test tube containing 300 µl of ice-cold "incubation buffer", centrifuged rapidly for two minutes at 13 000 rpm (Eppendorf centrifuge 3200). The supernatant was aspirated with a needle attached to a vacuum line and the pellet rinsed with 300 µl "incubation buffer". After the fluid was aspirated, 0.5 ml of NCS was added to the micro test tube which was then shaken and incubated overnight in an oven at 55°C. The next day, the contents of the tube were pipetted into a scintillation vial and the tube was rinsed with 0.5 ml of NCS which was also added to the vial. 7 ml toluene-PPO (0.7%) and 0.05 ml 10% ascorbic acid were added (to reduce chemiluminescence) and the vials counted in a two channel Beckman liquid scintillation counter (LS-250).

^{14}C-Sucrose was added to the assay medium to assess the amount of water present in the pellet after centrifugation and thus deduce the concentration of unbound (-)[^{3}H] dihydroalprenolol. Specific binding was taken as the difference (after correction from [^{14}C]-sucrose values) between total binding and binding in presence of 100 µM propranolol (non specific binding). Specific binding represented 30 to 60% of the total binding.

I. Lipid composition.

Lipids were extracted according to Kates (124). 3.2 ml of sarcolemma suspension in distilled water (4-6 mg/ml) was mixed with 8 ml of methanol and 4 ml of chloroform and then diluted with 4 ml of distilled water and 4 ml of chloroform. The two phases were separated by 2 minutes centrifugation (1000 rpm). The lower phase ($CHCl_3$) was collected while the upper phase (H_2O - CH_3OH) was extracted with 2 ml $CHCl_3$ and recentrifuged in a similar manner. The chloroform phases were pooled and completely dried under nitrogen flow. The residue was dissolved in 1 ml $CHCl_3$, concentrated again and redissolved in 0.1 ml $CHCl_3$. Fractionation of the lipid mixture into neutral lipids and phospholipids was done on a silicic acid column. 0.4-0.6 g of silicic acid (Bio-sil A, 100-200 mesh, dried in oven at 120°C overnight before the experiment) was placed in a 10 ml beaker with 2 ml $CHCl_3$. The solution was poured in a Pasteur pipette column blocked with glass wool. The sample (0.1 ml) was applied on the column. Neutral lipids were collected by elution with $CHCl_3$ (15 ml)

and phospholipids by elution with 10 ml of chloroform-methanol (1:1) and 25 ml of methanol. Total and free cholesterol were determined in the neutral fraction as described by Kates (124) (p360 - 361). The phospholipid sample was concentrated under a stream of nitrogen, dissolved in 1 ml $CHCl_3$, dried again, redissolved in 0.1 ml $CHCl_3$ and applied on a plate coated with silica gel H, together with appropriate phospholipid standards (PE, PC, PI, PS and sphingomyelin). After one hour development in $CHCl_3/CH_3OH/H_2O$ (65:35:5), the plate was stained with iodine (standards only). Each band was scraped into a glass-stoppered tube containing methanol-HCl (2.5% HCl in methanol) and heated at 70°C for one hour (four hours for sphingomyelin). After addition of 10% water, fatty acid methylesters were extracted with petroleum ether and analysed for each phospholipid by gas-liquid chromatography on a Carlo Erba fractovap (temperature: 185°C; column : 10% SP-2300 on 100/200 chromosorb WAW). Phosphorus was measured as described by Kates (124) (p 355; 4.2.2). Values for phospholipids were expressed as µmoles phospholipid by dividing the µg P obtained by 31.

J. Protein composition.

Polyacrylamide gel electrophoresis was done according to Weber et al., (232). The sarcolemma preparation (2 mg/ml) was diluted 1:1 with a solvent buffer containing 10 µM phenylmethylsulfonylfluoride (PMSF), heated at 100°C for two minutes and sonicated (Bronwill Biosonik III, micro-

probe, maximal intensity for 1.5 min). Plasma membranes were subsequently electrophoresed on 6% polyacrylamide gels in 0.1 M sodium phosphate buffer, pH 7.2, with 0.1% sodium dodecylsulfate (SDS) and 10 µM PMSF (40 µg protein per gel). Gels were stained with Coomassie Blue R250, destained in a diffusion destainer (BioRad Laboratories) and scanned at 540 nm (Gilford 2400 - 2).

K. Statistical analysis of results.

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

- unpaired: $$t = \frac{M_1 - M_2}{\sqrt{\varepsilon_1^2 + \varepsilon_2^2}}$$

M_1 & M_2 = means

ε_1 & ε_2 = standard errors

degree of freedom : $n_1 + n_2 - 2$

- paired: $$t = \frac{d}{\varepsilon}$$

d = mean of the differences between each pair

ε = standard error of the mean of the differences

degree of freedom : (number of pairs) - 1

CHAPTER IV: RESULTS AND DISCUSSION

In each section of this presentation and discussion of results, information is given about the two distinct components of the study:

(i) information obtained about purity, characteristics and integrity of the sarcolemmal preparation;

(ii) information obtained about differences in the characteristics of the sarcolemma in cold-acclimated (CA) rats as compared with warm-acclimated (WA) rats.

Results are usually presented in the following manner:

A. Purpose of the experiment.

B. Description of the experiment (supplementary details to those already described in chapter III)

C. Results and discussion.

PART 1 : YIELD OF PLASMA MEMBRANES.

The yield of plasma membranes is similar for muscles from WA rats and muscles from CA rats (Table II). However, the yield obtained in preparation II is significantly higher than in preparation I (about 35%). As far as technique I is concerned, the yield of sarcolemma compares quite well with data found in the literature: 0.5 and 0.672 mg/g muscle were reported respectively by Nagatomo & Peter (175) and Schapira

Table II. Yield of skeletal muscle sarcolemma isolated with techniques I and II (warm- and cold-acclimated rats).

	Technique I		Technique II
WA	0.42 ± 0.04	P < 0.05	0.57 ± 0.05
	NS		NS
CA	0.46 ± 0.03	P < 0.02	0.61 ± 0.05

Each result is the mean ± SEM of 13 preparations.
units are : mg protein/g wet weight muscle.

et al., (203) who used a strong salt extraction technique. Values for preparation II are higher than those reported by Kidwai et al., (127) and by Olefsky et al., (180) who used a sucrose density gradient technique (see Table I). This suggests either a better efficiency in preparation II or some contamination by other fractions.

PART 2 : MORPHOLOGY

A. Purpose of the study.

Technique I being different from other methods reported in the literature, it was necessary to study the morphology of sarcolemma isolated with this technique. The purpose was to assess the purity of the preparation and check for any difference between preparation from the WA and the CA rat.

B. Results.

Phase contrast photomicrographs of preparation I from WA and CA rats are shown in Fig. 18 and 19 respectively. In this preparation, sarcolemma has the appearance of empty tubes and looks similar to that isolated by Rosenthal et al., (199). Sarcolemma from the CA rat does not appear different from sarcolemma from the WA rat.

The preparations contained mainly sarcolemma, however, some contamination by mitochondria could be observed.

It was not possible to observe the morphological

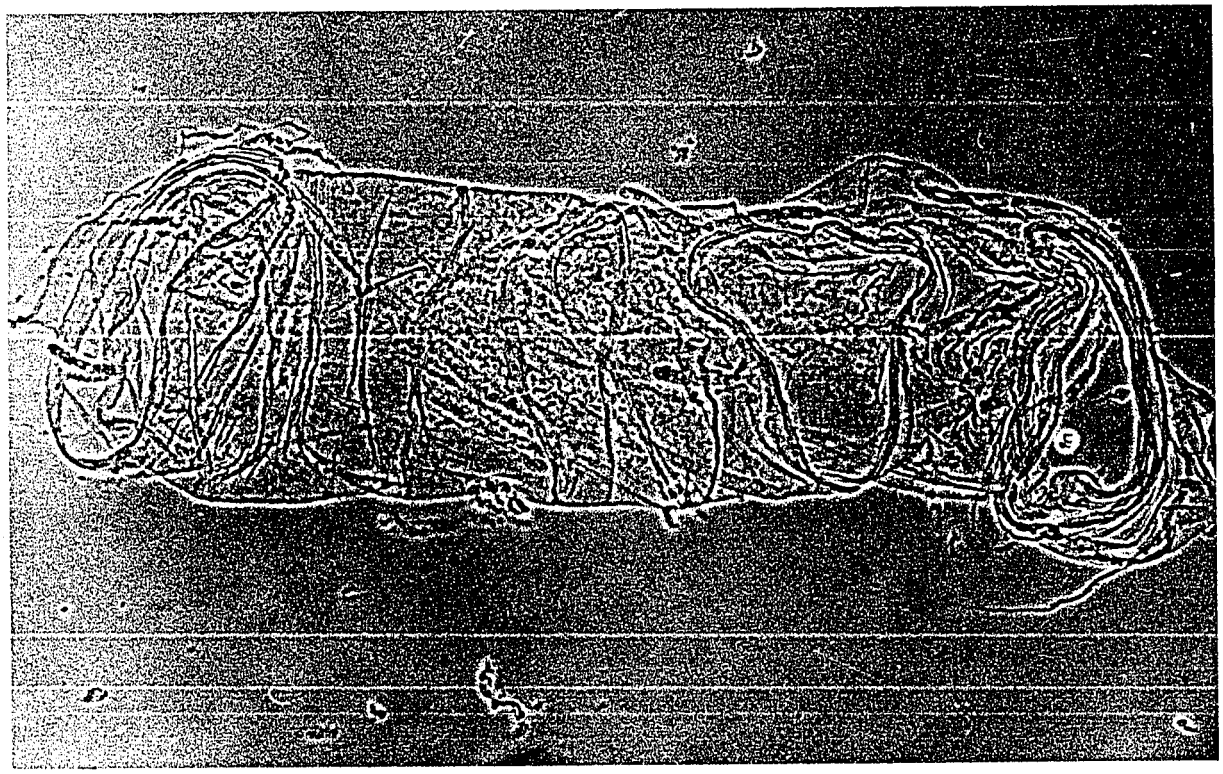

Fig. 18. Phase contrast photomicrograph of a sarcolemmal tube isolated with technique I (warm-acclimated rat).

microscope magnification : x 200

printing magnification : x 4.5

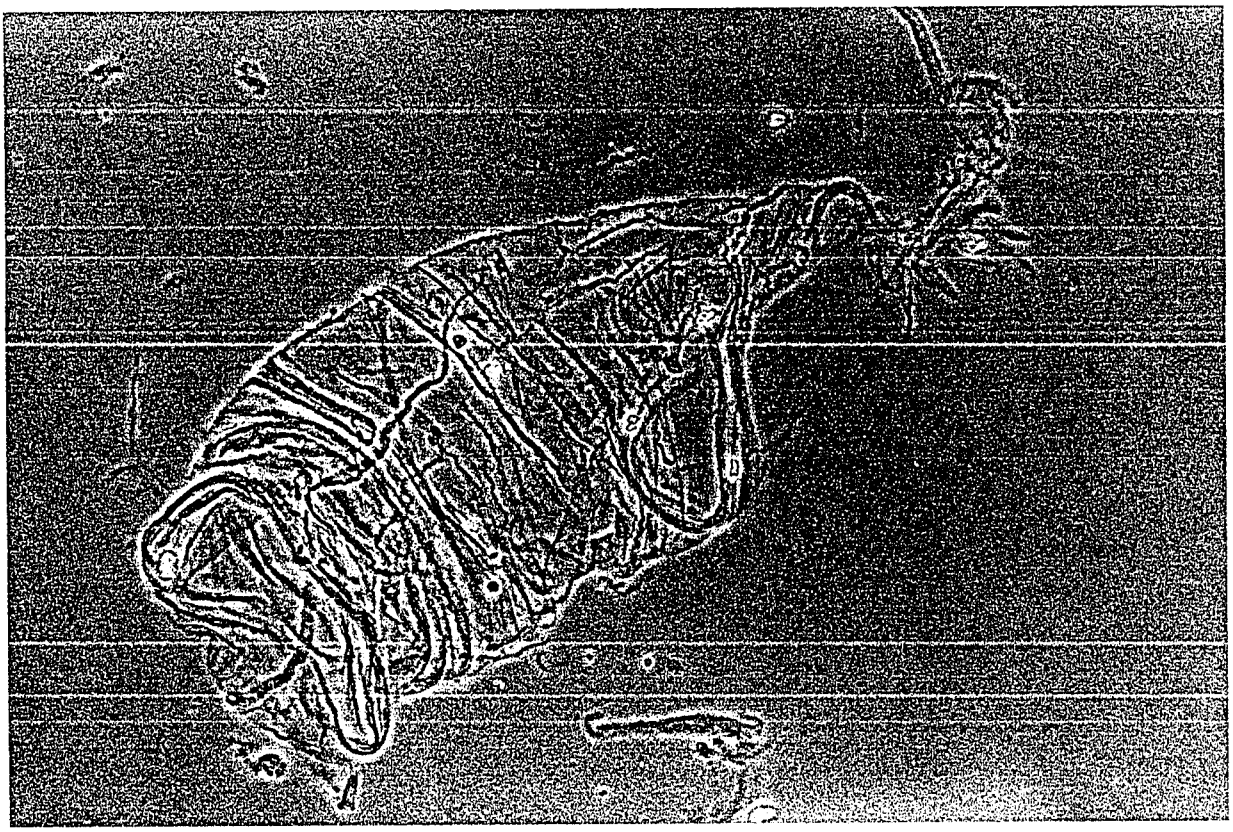

Fig. 19. Phase contrast photomicrograph of a sarcolemmal tube isolated with technique I (cold-acclimated rat).

microscope magnification : x 200

printing magnification: x 5.5

appearance of preparation II under phase contrast microscopy because of the small size of the material obtained; however, technique II being only slightly modified from that described by Kidwai et al., (127), there is no reason to believe that the result obtained differs from that reported by Kidwai et al., namely, the sarcolemma is isolated as small vesicles.

PART 3 : CYTOCHROME OXIDASE.

A. Purpose of the experiment.

The purpose was to assess any contamination of the preparations by mitochondria.

B. Description of the experiment.

4 WA rats (448 ± 24 g) and 4 CA rats (374 ± 14 g) were used to isolate preparation I, while 5 WA rats (463 ± 31 g) and 5 CA rats (393 ± 17) served for preparation II. 0.05 to 0.17 mg sarcolemmal protein was assayed as described under methods (III.2.D).

C. Results and discussion.

Activities observed vary from 0.29 to 0.56 µg atoms O_2/min.mg protein (Fig. 20). Values obtained for isolated muscle mitochondria are about 30 times higher (17), suggesting a contamination of about 3 to 6%. The two techniques (I & II) give preparations with similar and low contamination by mito-

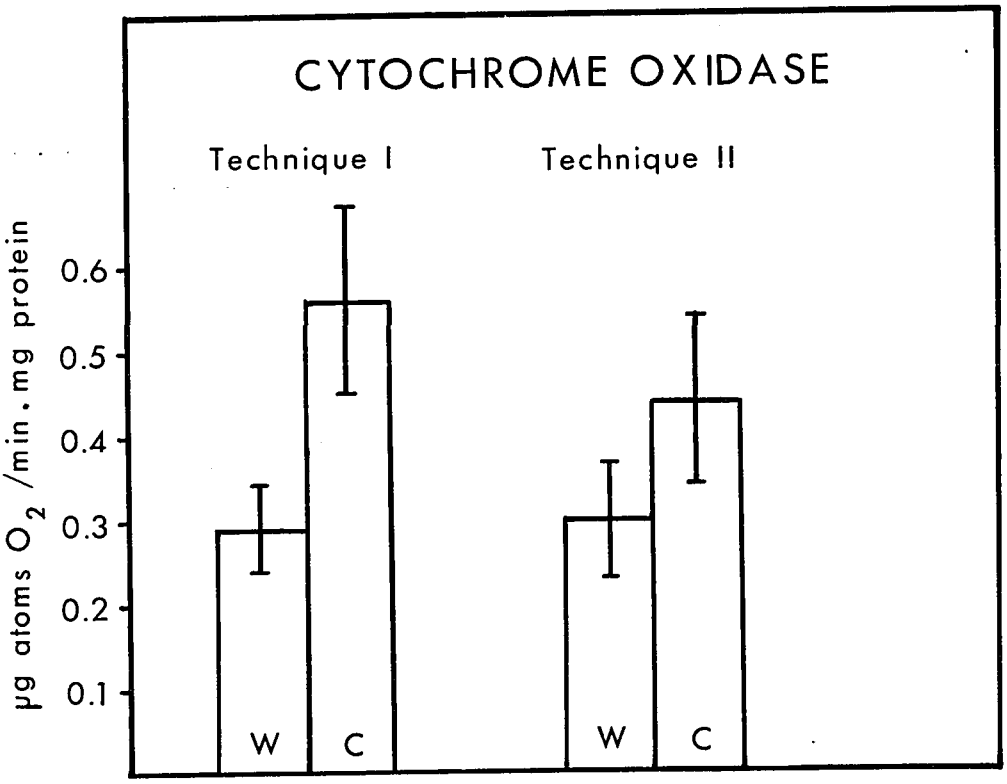

Fig. 20. Cytochrome oxidase activities in skeletal muscle sarcolemma isolated with techniques I and II (warm- and cold-acclimated rats).

Activities are expressed as mean ± SEM (µg atom O_2/min. mg protein). The following activities were obtained:

preparation I;	WA rats :	0.29 ± 0.05	NS (n = 4)
	CA rats :	0.56 ± 0.11	
preparation II:	WA rats :	0.30 ± 0.07	NS (n = 5)
	CA rats :	0.44 ± 0.10	

Abbreviations: W,WA : warm-acclimated
C,CA : cold-acclimated

legend continued on next page

The following table shows the cytochrome oxidase activities (μg atom O_2/min. mg protein) obtained for each experiment with preparations I and II. The means of the differences between values of each pair are also indicated.

Preparation I	WA	CA	
experiment			
1	0.22	0.51	
2	0.28	0.40	
3	0.43	0.50	
4	0.22	0.44	
	$d = 0.28 \pm 0.07$		$p < 0.05$

Preparation II			
experiment			
1	0.16	0.26	
2	0.19	0.40	
3	0.50	0.39	
4	0.21	0.82	
5	0.46	0.31	
	$d = 0.13 \pm 0.14$		NS

chondria. As far as cold acclimation is concerned, the cytochrome oxidase activity is higher in preparation I for the CA rat (paired t-test) (see Fig. 20). Mitochondria of skeletal muscle from CA rats are smaller and more numerous (17). Thus, it is possible that more mitochondria remain trapped in the sarcolemmal tubes from the CA rat. This interpretation does not apply to preparation II since the sarcolemmal tubes are not present in this preparation. The cytochrome oxidase activity present in preparation II is probably due to contamination by mitochondria damaged during the homogenization step, as reported by Kidwai et al., (127).

PART 4 : 5'-NUCLEOTIDASE.

A. Purpose of the experiment.

5'-Nucleotidase being a marker enzyme for sarcolemma, its activity was measured to check that sarcolemma was present in the preparations.

B. Description of the experiment.

For preparation I, 12 rats were used (6 WA and 6 CA). Their mean weights were: WA : 480 ± 10 g; CA : 389 ± 11 g. For preparation II, 20 rats were used (10 WA, 10 CA) with mean weights of: 407 ± 15 g (WA) and 329 ± 11 g (CA). 5'-Nucleotidase was assayed on isolated plasma membranes as described in methods (III.2.E.).

C. Results and discussion.

The results of the experiment are shown in Fig. 21. The activity of preparation I is about double the activity of

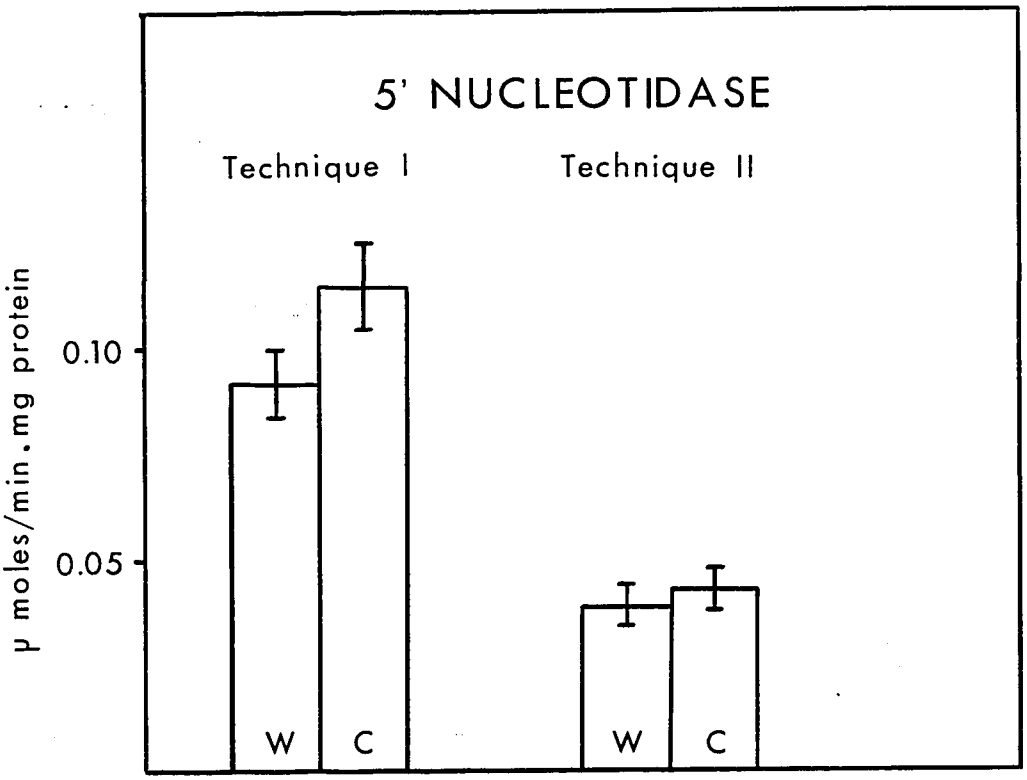

Fig. 21. 5'-Nucleotidase activities of skeletal muscle sarcolemma isolated with techniques I and II (warm- and cold-acclimated rats).

Activities are expressed as mean ± SEM (μmoles P_i/min. mg protein). Values are the following:

	WA		CA
preparation I	0.092 ± 0.008 (n = 6)	NS	0.115 ± 0.010 (n = 6)
	P < 0.001		P < 0.001
preparation II	0.036 ± 0.005 (n = 10)	NS	0.043 ± 0.005 (n = 10)

Abbreviations : W,WA : warm-acclimated
C,CA : cold-acclimated

legend continued on next page

The table below shows the 5'-nucleotidase activities (μmoles Pi/min. mg protein) obtained for each experiment with techniques I and II. The means of the differences between each pair are also indicated (± SEM).

Preparation I	WA	CA	
experiment			
1	0.078	0.100	
2	0.102	0.112	
3	0.096	0.146	
4	0.085	0.132	
5	0.069	0.120	
6	0.121	0.080	
	d = 0.023 ± 0.015		NS

Preparation II			
experiment			
1	0.045	0.031	
2	0.062	0.053	
3	0.042	0.053	
4	0.052	0.056	
5	0.021	0.038	
6	0.033	0.025	
7	0.021	0.035	
8	0.020	0.050	
9	0.042	0.036	
10	0.020	0.051	
	d = 0.007 ± 0.005		NS

preparation II.

With a technique similar to technique I, Severson et al., (207) could not detect any 5'-nucleotidase activity in rabbit skeletal muscle sarcolemma, explained by them as being due to solubility of the enzyme in their preparation. However, the value reported in this thesis compares quite well with results obtained by Schapira et al., (203) who used a technique similar to technique I. The fact that their value is somewhat higher might be explained by their further purification of the sarcolemma on sucrose density gradient after the salt extraction. For preparation II, the activity is about half of that obtained by Kidwai et al., (127). It is also lower than that reported by Olefsky et al., (180) who used a similar isolation technique (see table I).

Homogenization in technique II appears to be very critical and it is thus possible that the composition of Kidwai's preparation and preparation II might be different. In particular, contamination by sarcoplasmic reticulum in preparation II might be greater, thus reducing 5'-nucleotidase activity. The discrepancy could also be the effect of different methods to assess 5'-nucleotidase activity. 5'-Nucleotidase has been reported to be located at the external surface of the cell (239). It is thus possible that the accessibility of the substrate (AMP) to the enzyme is impaired if the plasma membrane vesicles are inside-out in preparation II. Further evidence that this explanation may , in fact, be correct will be provided later in this thesis.

As far as cold-acclimation is concerned, there is no statistically significant difference between the WA and the CA rat in either preparation (assessed with both unpaired and paired t-tests).

PART 5 : Na,K-ATPase.

A. Purpose of the experiment.

This experiment had a dual purpose. First, Na,K-ATPase activity was measured as a marker for plasma membrane (see section II.3.A and II.4.B.ii). The second purpose was to check whether acclimation to cold induces an adaptive change in the activity of the Na,K-ATPase in skeletal muscle. If Na,K-ATPase contributes to the enhanced calorigenic response to catecholamine administration observed in the CA rat, one might expect its activity to be increased in the CA rat and probably influenced by the presence of catecholamines.

B. Description of the experiment.

For preparation I : 8 WA rats weighing 492 ± 14 g
8 CA rats weighing 406 ± 16 g

For preparation II: 3 WA rats weighing 512 ± 11 g
3 CA rats weighing 410 ± 8 g

ATPase activities were measured as described in methods (III.2.F.). The following activities were assayed:

Total ATPase: ATPase activity in presence of Na^+,$K^+$and Mg^{++}.

Mg-ATPase: ATPase activity in presence of Na^+,K^+, Mg^{++} and ouabain (1 - 2 mM) or in presence of Mg^{++} and choline

chloride (120 mM). Na,K-ATPase represents the difference between these two activities (total ATPase minus Mg-ATPase).

4 mM tris-ATP was used for preparation I and 4 mM Na-ATP (vanadium-free) started the incubation of preparation II. The influence of various compounds such as adrenaline, noradrenaline and cyclic AMP was studied; their concentration was 10^{-4}M.

C. Results and discussion.

Fig. 22 and Fig. 24 show, respectively for preparation I and II, the activities measured in presence of Na^+,K^+ and Mg^{++} (total), when ouabain is present in the assay (ouabain) or when NaCl and KCl are replaced by choline chloride ($-Na^+$ $-K^+$). Each figure shows the results obtained for the WA and the CA rats. Fig. 23 and Fig. 25 illustrate the data presented in Fig. 22 and Fig. 24 respectively, but so that the activity of Na,K-ATPase is more clearly visible. Na,K-ATPase has been expressed as the difference between activity of total ATPase and ATPase activity measured with choline chloride in the assay instead of NaCl + KCl. The remainder of the total ATPase activity is the Mg-ATPase activity. Finally, values of Na,K-ATPase activities for the WA and the CA rats in both preparations (I and II) have been gathered in Fig. 26 to make the comparison easier.

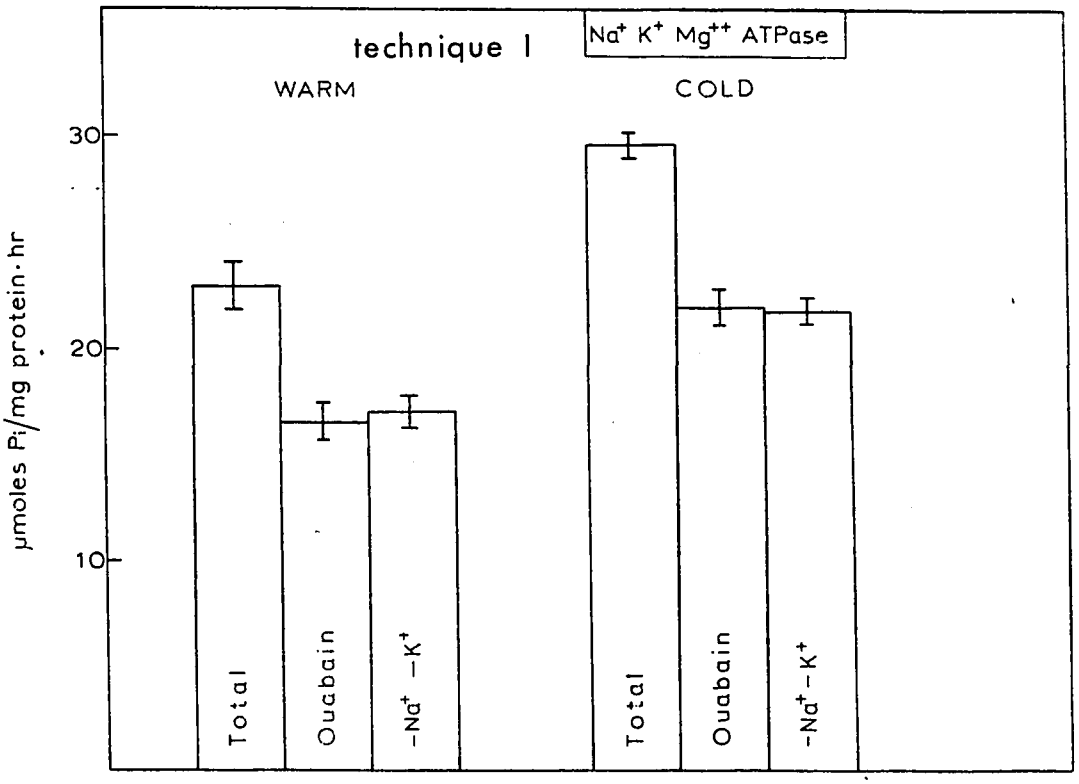

Fig. 22. ATPase activities of skeletal muscle sarcolemma isolated with technique I (warm- and cold-acclimated rats).

Activities (µmoles P_i/mg protein.hr) were measured as described in methods with tris-ATP as substrate. For each set of 3 bars, the bar on the left represents the total ATPase activity measured in presence of Na^+,K^+ and Mg^{++}. In the assay corresponding to the bar in the middle, ouabain (1 mM) was added whereas on the right, NaCl and KCl were replaced by choline chloride.

Values obtained are from left to right (means ± SEM of 8 experiments):

WA rats:	22.9 ± 1.1	16.5 ± 0.9	17.0 ± 0.8
	P < 0.001	P < 0.001	P < 0.001
CA rats:	29.6 ± 0.6	22.0 ± 0.8	21.8 ± 0.7

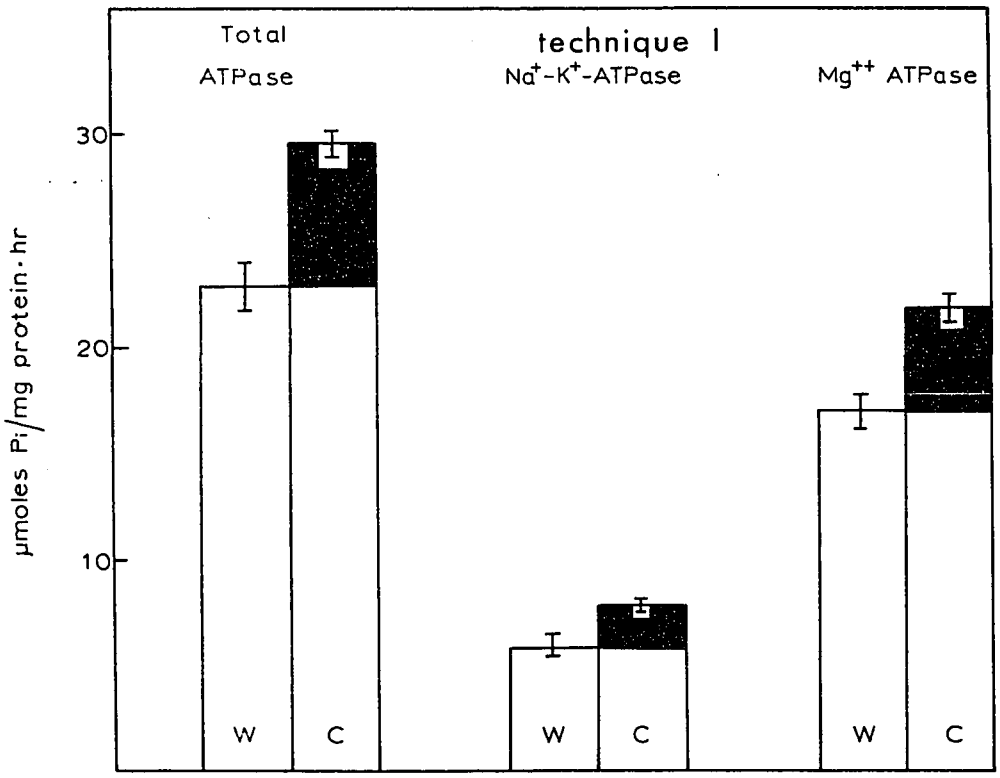

Fig. 23. Na,K-ATPase and Mg-ATPase activities of skeletal muscle sarcolemma isolated with technique I (warm- and cold-acclimated rats).

Data are those presented in Fig. 22 but in a different manner. Na,K-ATPase activity has been taken as the difference between total ATPase activity (left) and activity when NaCl and KCl were replaced by choline chloride (right). Values (means ± SEM) for warm- (W) and cold-(C) acclimated rats are from left to right (n = 8):

	W	C	
total ATPase	22.9 ± 1.1	29.6 ± 0.6	P < 0.001
Na,K-ATPase	5.9 ± 0.5	7.8 ± 0.3	P < 0.01
Mg-ATPase	17.0 ± 0.8	21.8 ± 0.6	P < 0.001

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The following table shows the results obtained for total ATPase and Mg ATPase in each experiment. (μmoles P_i/ mg protein.hr)

	Total ATPase		Mg^{++}-ATPase	
Experiment	W	C	W	C
1	21.0	30.2	15.5	21.1
2	26.6	26.4	18.7	19.3
3	20.8	30.4	14.9	22.6
4	19.0	30.6	15.2	23.9
5	19.7	28.9	14.6	20.3
6	25.2	31.6	20.5	24.0
7	26.0	27.7	18.5	20.1
8	25.2	31.3	18.4	23.2

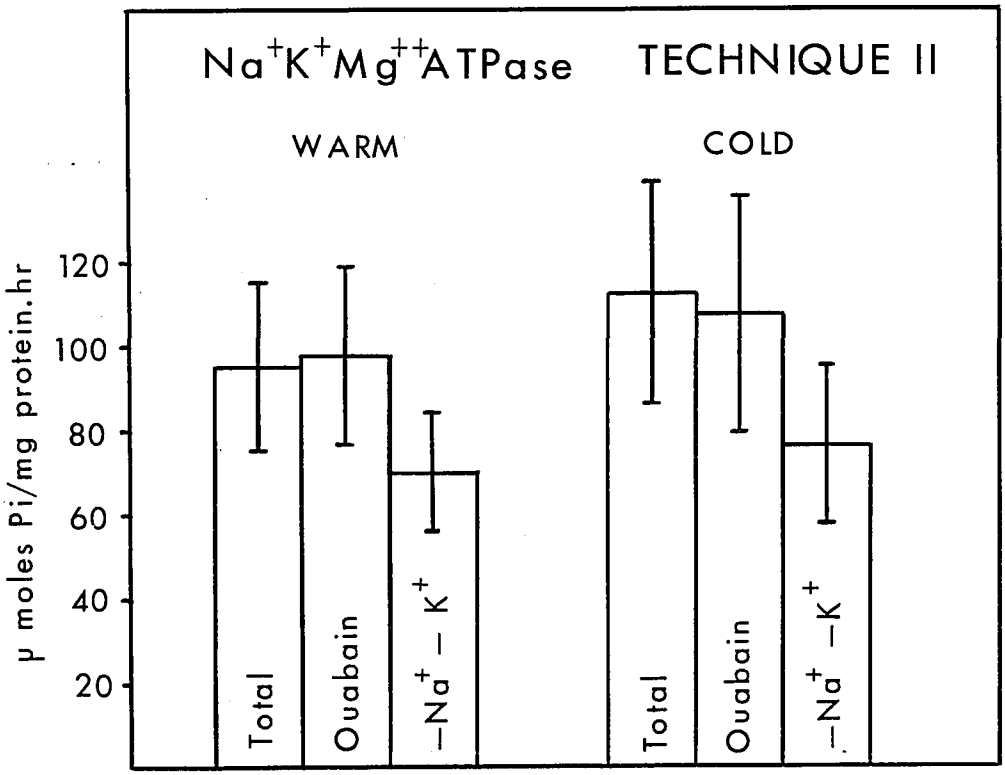


Fig. 24. ATPase activities of skeletal muscle sarcolemma isolated with technique II (warm- and cold-acclimated rats).

Activities (μmoles P_i/mg protein.hr) were measured as described in methods with Na-ATP (vanadium-free) as substrate. For each set of 3 bars, the bar on the left represents the total ATPase activity measured in presence of Na^+, K^+ and Mg^{++}. In the assay corresponding to the bar in the middle, ouabain (2 mM) was added whereas on the right, NaCl and KCl were replaced by choline chloride.

Values obtained are from left to right (means ± SEM of 3 experiments):

WA rats :	95.4 ± 20.1	98.0 ± 21.3	70.0 ± 14.0
	NS	NS	NS
CA rats :	112.5 ± 26.5	107.5 ± 28.2	76.7 ± 18.7

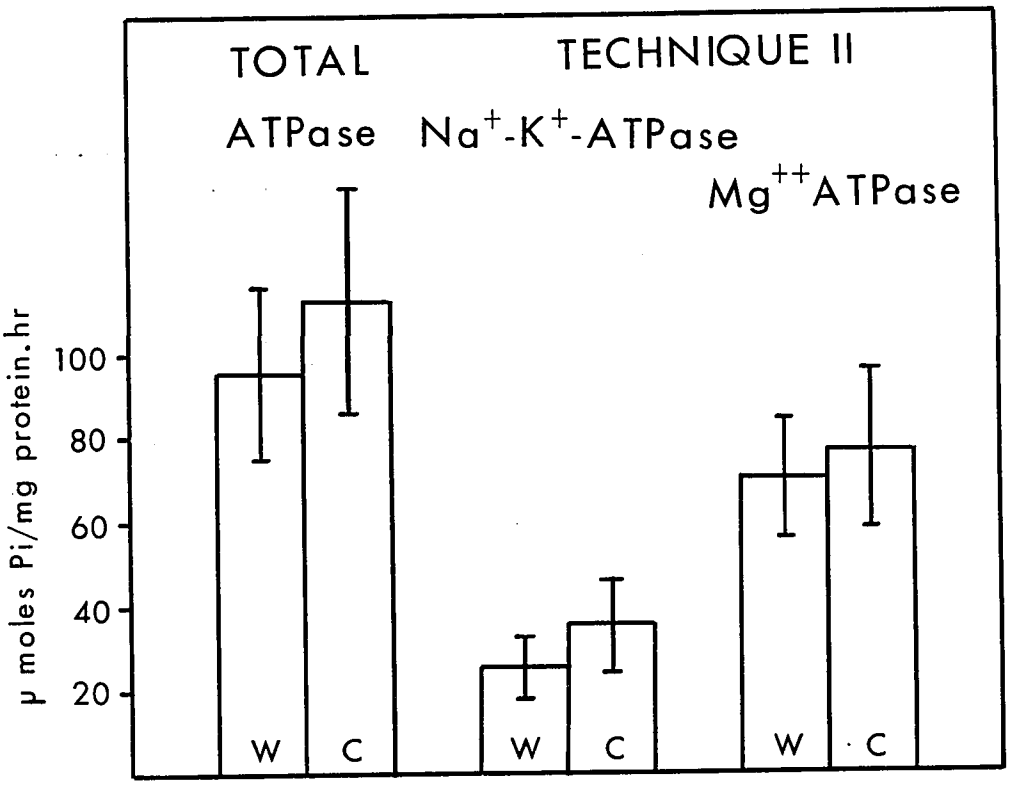


Fig. 25. Na,K-ATPase and Mg-ATPase activities of skeletal muscle sarcolemma isolated with technique II (warm- and cold-acclimated rats).

Data shown are those presented in Fig. 24 but in a different manner. Na,K-ATPase activity has been taken as the difference between total ATPase activity (left) and activity when NaCl and KCl were replaced by choline chloride (right). Values (means ± SEM) for warm- (W) and cold - (C) acclimated rats are from left to right (n = 3):

	W	C	
total ATPase:	95.4 ± 20.1	112.5 ± 26.5	NS
Na,K-ATPase:	25.4 ± 7.3	35.9 ± 9.9	NS
Mg-ATPase:	70.0 ± 14.0	76.7 ± 18.7	NS

legend continued on next

Legend to Figure 25, continued.

The following table shows the results obtained for total ATPase and Mg ATPase in each experiment (μmoles P_i/ mg protein.hr)

	Total ATPase		Mg^{++}-ATPase	
Experiment	W	C	W	C
1	79.2	92.6	64.8	72.4
2	135.4	165.0	96.3	110.9
3	71.5	80.0	48.8	46.7

Fig. 26. Na,K-ATPase activities of skeletal muscle sarcolemma isolated with techniques I and II (warm- and cold-acclimated rats).

Data presented are taken from Fig. 23 and 25. Values are means ± SEM (μmoles/mg protein.hr):

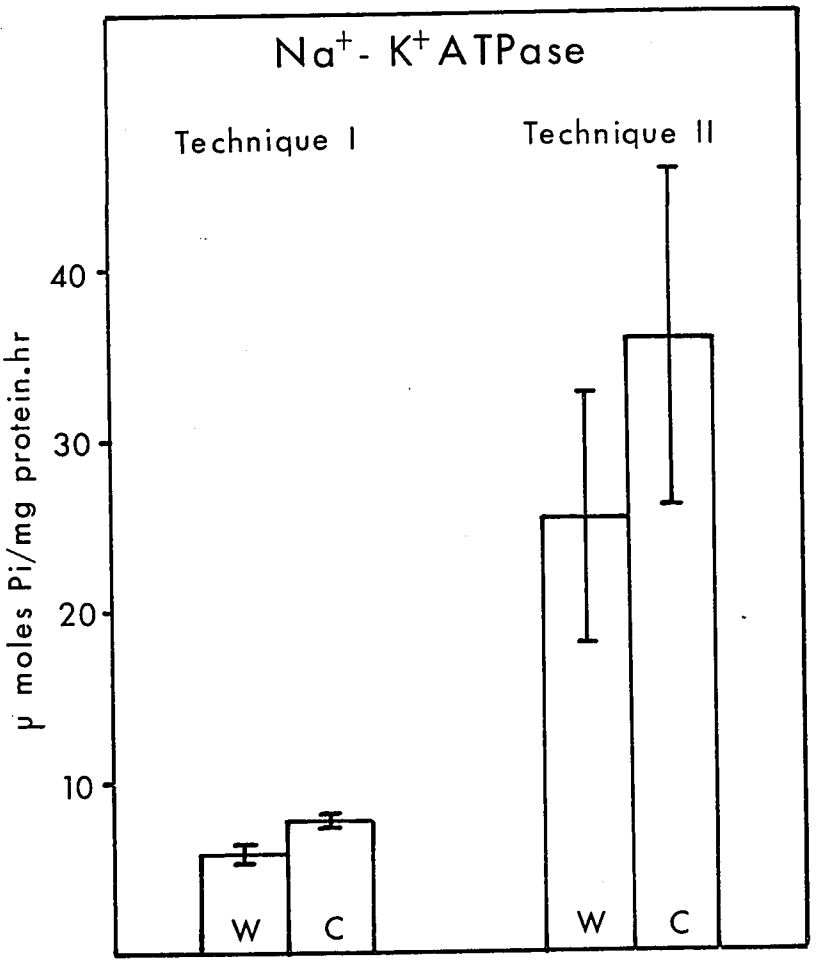
	Warm (W)		Cold (C)	
technique I	5.9 ± 0.5	P < 0.01	7.8 ± 0.3	(n = 8)
technique II	25.4 ± 7.3	NS	35.9 ± 9.9	(n = 3)

The table below shows the Na,K-ATPase activities obtained for each experiment with technique I.

Experiment	Warm	Cold
1	5.5	9.1
2	8.0	7.1
3	5.9	7.8
4	3.9	6.7
5	5.1	8.6
6	4.7	7.6
7	7.5	7.6
8	6.8	8.2

The table below shows the Na,K-ATPase activities obtained for each experiment with technique II. The percentage increase from the WA to the CA rat and its mean ± SEM are also indicated.

Experiment	Warm	Cold	% increase
1	14.4	20.2	40.3
2	39.1	54.1	38.4
3	22.7	33.3	46.7
			41.8 ± 2.5
			(P < 0.005)


Na+- K+ ATPase
Technique I
Technique II
μ moles Pi/mg protein.hr
40
30
20
10
W
C
W
C

These results will be discussed at two levels:

- characterization of sarcolemma
- role of Na,K-ATPase in cold acclimation.

C(i). Characterization of sarcolemma.

Na,K-ATPase activity of sarcolemma isolated with technique I is lower (5.9 µmoles/mg protein.hr) than activity in preparation II (25.4 µmoles/mg protein.hr). This appears to be a general characteristic as indicated by the literature. For example, values of 6 and 11 µmoles/mg protein.hr have been reported for rat muscle respectively by Nagatomo & Peter (175) and Schapira et al., (203) who used high salt extraction similar to technique I. On the other hand, values of 15 and 31 µmoles/mg protein.hr have been reported respectively by Kidwai et al., (127) and Barchi et al., (10) who extracted plasma membranes on sucrose density gradient. One drawback of technique II is the high activity of Mg-ATPase (70 ± 14 moles/mg protein.hr) which tends to mask the Na,K-ATPase activity. (This high Mg-ATPase activity was also present in Kidwai's and in Barchi's preparations).

The high value obtained for Na,K-ATPase in preparation II is not only due to the extraction conditions but also to the incubation conditions. Indeed, vanadium-free ATP was used for this assay. The presence of an inhibitor of Na,K-ATPase in Sigma grade ATP has been known for some time (36). It is only recently that this inhibitor has been identified

as vanadate (30) and the inhibition studied (15,16,31). In one experiment, the influence of the source of ATP on the activity of Na,K-ATPase in preparation II was studied. The following activities (μmoles/mg protein.hr) were found, using respectively tris-ATP, Na-ATP (Sigma grade, containing vanadium) and Na-ATP (vanadium-free) : 12.3; 14.4; 20.2. These data mean that when tris-ATP or Na-ATP (Sigma grade) are used, Na,K-ATPase is inhibited by 30 to 40% by vanadium present in the substrate. However the value found for preparation II (see Fig. 26), even if reduced by 30 to 40% still compares quite well with data reported in the literature (10,127).

Preparations I and II behave differently with respect to ouabain inhibition. In preparation I (Fig. 22) 100% inhibition of Na,K-ATPase activity is obtained with 1 mM ouabain, whereas 2 mM ouabain does not cause any inhibition of the enzyme in preparation II (Fig. 24). One hypothesis, already put forward for 5'-nucleotidase, is that the plasma membrane vesicles obtained with preparation II could be inside-out, thus preventing ouabain access to its binding site known to be on the outer surface of the membrane (93). This hypothesis is not contradicted by reports in the literature: Kidwai and coworkers (127) used a high concentration of ouabain (3 mM) to inhibit the Na,K-ATPase (usual concentrations vary from 0.1 mM to 1 mM); Barchi and coworkers (10) reported an incomplete inhibition of Na,K-ATPase activity by ouabain.

This hypothesis of inside-out vesicles can also be put forward to explain the fact that the Na,K-ATPase activity is higher in the preparation where 5'-nucleotidase activity is lower, namely preparation II (see Fig. 21 & 26). Na,K-ATPase, contrary to 5'-nucleotidase, is an intrinsic component of the membrane. The binding site for the substrate (ATP) is located on the inner face of the membrane (93). Thus, in inside-out vesicles, ATP would have better access to its binding site. This is an explanation of the higher Na,K-ATPase activity observed in preparation II. The possibility still exists, however, that Na,K-ATPase is inactivated in preparation I by the strong salt concentrations which are characteristic of technique I.

Lubrol has been shown to unmask Na,K-ATPase sites in cardiac sarcolemmal vesicles, the overall effect being a 3.5-fold increase in Na,K-ATPase activity (19). The effect of lubrol was tested in preparation II in one experiment. Instead of the expected increase in Na,K-ATPase activity, a decrease in both Mg-ATPase and Na,K-ATPase activities was observed. However, some inhibition by ouabain was present in the preparation. These results also confirm the hypothesis of inside-out vesicles: lubrol in such vesicles would permit access of ouabain to its binding site but reduce the activity of the enzyme.

In conclusion, it appears that comparison of preparations I and II is difficult. The difficulty resides in the fact that the preparations differ considerably

at the morphological level and in the procedure used to obtain the final result. However, both preparations (I & II) clearly contain plasma membranes, as shown by their Na,K-ATPase and 5'-nucleotidase activities.

C(ii). Role of Na,K-ATPase in cold acclimation.

As indicated in Fig. 26, a significant increase (32%) in activity of Na,K-ATPase is observed in the CA rat for preparation I. This increase, is also seen in preparation II (42%), although not significant. The increase in Na,K-ATPase in preparation II is not significant for two reasons: small number of experiments (n = 3) and high variation from one experiment to the other. However, the percentage increase in Na,K-ATPase activity in the CA rats is very reproducible (41.8 ± 2.5%) as indicated by the data from each experiment given in Fig. 26. ($P < 0.005$)

A 33% increase in activity is present also for Mg-ATPase in preparation I (Fig. 23). However, the increase is not significant in preparation II (Fig. 25).

Thus it seems that sarcolemma from CA rats differs from that isolated from WA rats.

However, no influence of noradrenaline (10^{-4}M) or adrenaline (10^{-4}M) on Na,K-ATPase was detected, either in the WA or the CA rat sarcolemmal preparations. A possibility was that the receptor sites had been damaged during the extraction. To bypass any receptor damage, the effect of cyclic AMP was tested directly : no action of cyclic AMP was observed. For this reason, the number of receptor sites

was measured directly (see section IV.7.). Another explanation for the lack of effect of catecholamines is that a link was missing between the catecholamine receptor site (or cyclic AMP) and the Na,K-ATPase. However, addition of protein kinase to assays containing noradrenaline (or cyclic AMP) did not produce any change in Na,K-ATPase activity.

As already mentioned (sections II.2.B.ii and II.3.A.iii), regulation of Na,K-ATPase by catecholamines is complex. Activation of Na,K-ATPase can proceed through reversal of inhibition. In fact, the inhibition of Na,K-ATPase by vanadium, mentioned above, has been reported to be reversed by catecholamines (30,105,122). The lack of effect of noradrenaline on vanadium inhibition in preparation I is probably due to the low noradrenaline concentration used. Noradrenaline concentrations reported to reverse the inhibition by vanadium were 10 to 25 times higher (105,122). In brain, activation of Na,K-ATPase by catecholamines has been shown to be indirect too, and proceed through chelation by catecholamines of ions such as Ca^{++} (70) or Fe^{++} (83). These divalent cations being strong inhibitors of Na,K-ATPase, their removal leads to activation of the enzyme. The presence of EGTA in the incubation medium prevents this indirect activation of Na,K-ATPase (70). Assays of preparations I & II contained EGTA. This is a probable explanation of the lack of action of catecholamines on Na,K-ATPase. However, activation of

Na,K-ATPase by catecholamines was reported with skeletal muscle sarcolemma incubated with EGTA (37).

Thus, any conclusion about the mode of action of catecholamines on Na,K-ATPase is difficult to draw; this only confirms the inconsistency of the data reported in the literature (13,148,157).

As far as cold acclimation is concerned, the observed increase in Na,K-ATPase activity in sarcolemma of the cold-acclimated rat might indicate an increased operation of this enzyme in the intact animal. This would be in keeping with the known increase in ouabain-sensitive tissue respiration described above (section II.2.B.ii). However, the lack of any demonstrable stimulatory effect of catecholamines on the enzyme in isolated sarcolemma would suggest that if the enzyme does participate in nonshivering thermogenesis in vivo, then the regulation of its activity by catecholamines must be indirect. Since catecholamines do apparently increase Na^+ and K^+ transport in intact muscle (41,198), it might be postulated that they act by altering permeability to these ions. A different action in the cold-acclimated rat would then have to be ascribed to an altered action on cell permeability, not detectable in the isolated sarcolemma used in these experiments.

PART 6 : ADENYLATE CYCLASE.

A. Purpose of the experiment.

As mentioned above, adenylate cyclase has already been studied on skeletal muscle homogenates from CA and WA rats (170). Although a transient increase in basal, fluoride-stimulated and noradrenaline-stimulated adenylate cyclase activity was observed during acclimation to cold, no change was detected in the adenylate cyclase system of CA rats. But, if this enzyme is involved in the enhancement of the metabolic response to noradrenaline during cold-acclimation, one might expect the enzyme to be regulated differently in the WA and the CA animals. For this reason, it appeared interesting to study the stimulation of the enzyme by GppNHp with and without noradrenaline. Moreover, the advantage of sarcolemma versus homogenate is that any change would be more readily detected in a sarcolemmal preparation.

B. Description of the experiment.

For preparation I, 4 CA rats and 4 WA rats weighing respectively 410 ± 31 g and 456 ± 30 g were used, whereas with technique II, plasma membranes were extracted from muscles of 7 CA rats (335 ± 13 g) and 7 WA rats (434 ± 22 g). Adenylate cyclase was assayed as described in methods (III.2.G.).

The following activities were measured:

- basal
- stimulation by NaF (8 mM)
- stimulation by GppNHp (10^{-4}M)
- stimulation by noradrenaline (10^{-4}M)
- combined stimulation by GppNHp and noradrenaline.

C. Results and discussion.

Fig. 27 and 28 show the results obtained. They are similar in both preparations:

- low basal activity.
- stimulation by NaF 12 to 20 times depending on the preparation.
- large stimulation by GppNHp (40 to 50 times the basal activity).
- no effect of noradrenaline.
- large stimulation by GppNHp combined with noradrenaline.

The basal activity is about the same as the activity measured in homogenates (170). This probably reflects some inactivation of the enzyme due to sarcolemma extraction techniques. Lack of catecholamine stimulation has already been reported for example in human fat cells (44). No synergistic action of GppNHp combined with noradrenaline is observed. This is consistent with the lack of effect of noradrenaline alone.

Values for preparation I are about 65% those obtained for preparation II. Here again, the hypothesis of inside-out

Fig. 27. Adenylate cyclase activities of skeletal muscle sarcolemma isolated with technique I (warm- and cold-acclimated rats).

The graph compares the activities of adenylate cyclase in preparation I from warm- (W) and cold- (C) acclimated rats. The bars show the means obtained (± SEM) and signify from left to right : basal activity, incubation with NaF (8 mM), GppNHp (10^{-4}M), noradrenaline (10^{-4}M) and both GppNHp and noradrenaline. Values are the following:

	WA (n = 4)	CA (n = 4)	
basal	3.5 ± 1.4	4.3 ± 1.6	NS
8 mM NaF	57.0 ± 7.5	51.7 ± 9.6	NS
10^{-4}M GppNHp	145.5 ± 13.7	175.0 ± 25.4	NS
10^{-4}M NA	5.4 ± 2.2	7.1 ± 4.3	NS
GppNHp + NA	148.7 ± 16.9	187.9 ± 28.7	NS

Units are : pmoles cyclic AMP/min.mg protein

Abbreviations: GppNHp: 5'-guanylyl imidodiphosphate

NA : noradrenaline

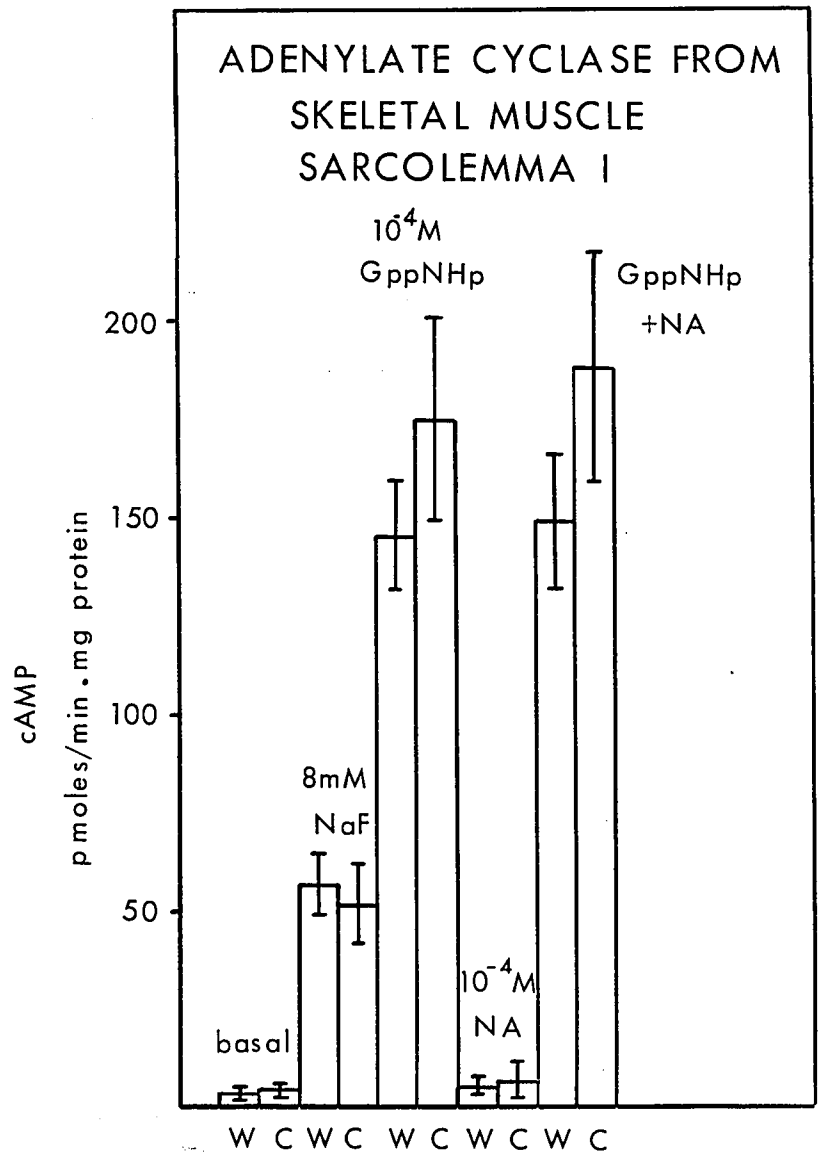

ADENYLATE CYCLASE FROM
SKELETAL MUSCLE
SARCOLEMMA I
cAMP
pmoles/min.mg protein
200
150
100
50
basal
8mM
NaF
10⁻⁴M
GppNHp
10⁻⁴M
NA
GppNHp
+NA
W C W C W C W C W C

Fig. 28. Adenylate cyclase activities of skeletal muscle sarcolemma isolated with technique II (warm- and cold-acclimated rats).

The graph compares the activities of adenylate cyclase in preparation II from warm- (W) and cold- (C) acclimated rats. The bars show the means obtained (± SEM) and signify from left to right : basal activity, incubation with NaF (8 mM), GppNHp (10^{-4}M), noradrenaline (10^{-4}M) and both GppNHp and noradrenaline. Values are the following:

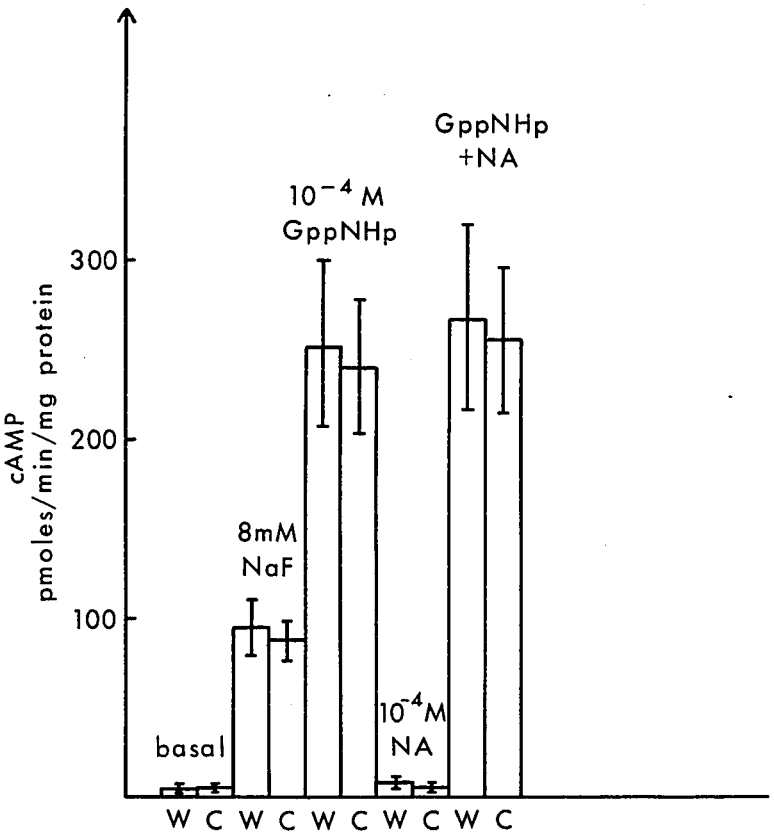
	WA (n = 7)	CA (n = 7)	
basal	4.7 ± 1.0	5.1 ± 0.3	NS
8 mM NaF	94.9 ± 15.5	88.0 ± 10.9	NS
10^{-4}M GppNHp	251.8 ± 46.2	241.5 ± 36.9	NS
10^{-4}M NA	7.7 ± 1.0	6.0 ± 1.2	NS
GppNHp + NA	268.0 ± 53.1	256.0 ± 41.8	NS

Units are : pmoles cyclic AMP/min.mg protein

Abbreviations: GppNHp : 5'-guanylyl imidodiphosphate

NA : noradrenaline

ADENYLATE CYCLASE FROM SKELETAL MUSCLE SARCOLEMMA II



vesicles can be put forward : the higher activity of preparation II could be due to better accessibility of the substrate (ATP) to the enzyme.

An important observation is that there is no difference between the WA and the CA rats. This confirms the results found in homogenates by Muirhead & Himms-Hagen (170).

In conclusion, the enhanced calorigenic response to noradrenaline administration observed in the cold-acclimated rat does not seem to be due to an alteration in the response of adenylate cyclase to this compound. If any altered regulation by guanyl nucleotides exists in vivo, it is however not visible in vitro.

PART 7 : β-ADRENERGIC RECEPTORS.

A. Purpose of the experiment.

Because of the lack of responsiveness of both preparations to catecholamines, it was important to check whether the receptors for catecholamines had been preserved during the extraction procedure. Moreover, if any presence of these receptors were demonstrable, one would expect their number to be smaller in the CA animal (desensitization due to high concentration of catecholamines) and this number to come back to control level upon addition of GppNHp (resensitization by guanyl nucleotides) (see section II.3.B.).

B. Description of the experiment.

Skeletal muscle plasma membranes from 5 WA rats weighing 480 ± 12 g and 5 CA rats weighing 386 ± 13 g were prepared with technique I. For preparation II, 3 WA rats (512 ± 11 g) and 3 CA rats (410 ± 8 g) were used. The number of β-adrenergic receptors was assessed by (-)[^{3}H]dihydroalprenolol binding as described in methods (III.2.H.).

C. Results and discussion.

Fig. 29 & 30 illustrate the results obtained for preparations I and II respectively. The results are similar in both preparations and show the presence of specific binding of (-)[^{3}H]DHA. It appears surprising at first, that specific binding occurs to the same extent in preparations I and II since the vesicles prepared with technique II are presumably inside-out. It is possible however that in neither preparation does the level of specific binding of (-)[^{3}H]DHA correspond to the true number of receptors : some damage to the receptors might occur with technique I thus decreasing the concentration of bound (-)[^{3}H]DHA whereas in preparation II, this decrease might be due to the fact that most of the vesicles are inside-out. This is an hypothetical explanation for the similarity of binding data in both preparations. As no binding data for skeletal muscle are available in the literature, it is however not possible to answer the question about the true number of β-adrenergic receptors.

It appears that binding of (-)[^{3}H]DHA is decreased in the CA rat in both preparations (46% and 45% decrease in pre-

Fig. 29. (-)[^{3}H]Dihydroalprenolol binding to skeletal muscle sarcolemma isolated with technique I (warm- and cold-acclimated rats).

The figure compares (-)[^{3}H] dihydroalprenolol binding to sarcolemma from warm- and cold-acclimated rats, with and without GppNHp (0.12 mM) in the incubation medium. The bars are the means ± SEM of specific binding, i.e. the differences between total binding and binding in presence of propranolol (100 µM). Values are the following (pmoles/mg protein) (n = 5):

	W		C
without GppNHp	0.446 ± 0.145	NS	0.224 ± 0.098
	NS		NS
with GppNHp	0.415 ± 0.120	NS	0.390 ± 0.116

The following table indicates the values obtained for binding of (-)[^{3}H] dihydroalprenolol in each experiment for the warm- and cold-acclimated rats with and without GppNHp.

Experiment	WA	CA	CA + GppNHp	WA + GppNHp
1	0.379	0.119	0.209	0.273
2	0.486	0.089	0.559	0.665
3	0.980	0.540	0.716	0.694
4	0.231	0.358	0.399	0.383
5	0.153	0.013	0.070	0.060

Abbreviations:
W: warm-acclimated
C: cold-acclimated
GppNHp: 5'-guanylyl imidodiphosphate

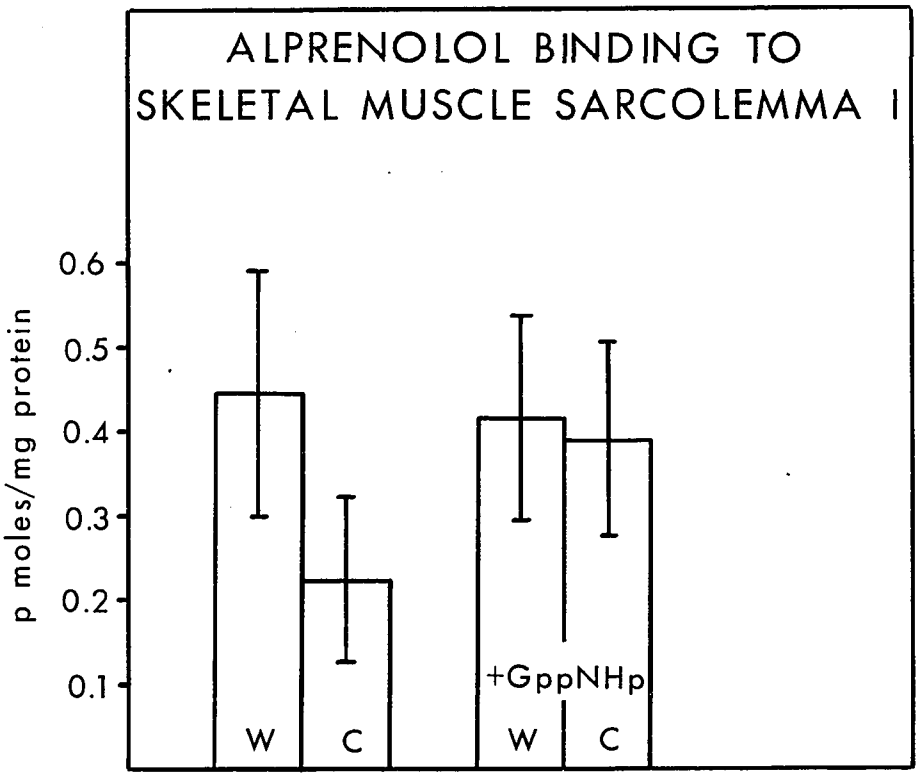

ALPRENOLOL BINDING TO
SKELETAL MUSCLE SARCOLEMMA I
p moles/mg protein
0.6
0.5
0.4
0.3
0.2
0.1
W
C
+GppNHp
W
C

Fig. 30. (-)[^{3}H]Dihydroalprenolol binding to skeletal muscle sarcolemma isolated with technique II (warm- and cold-acclimated rats).

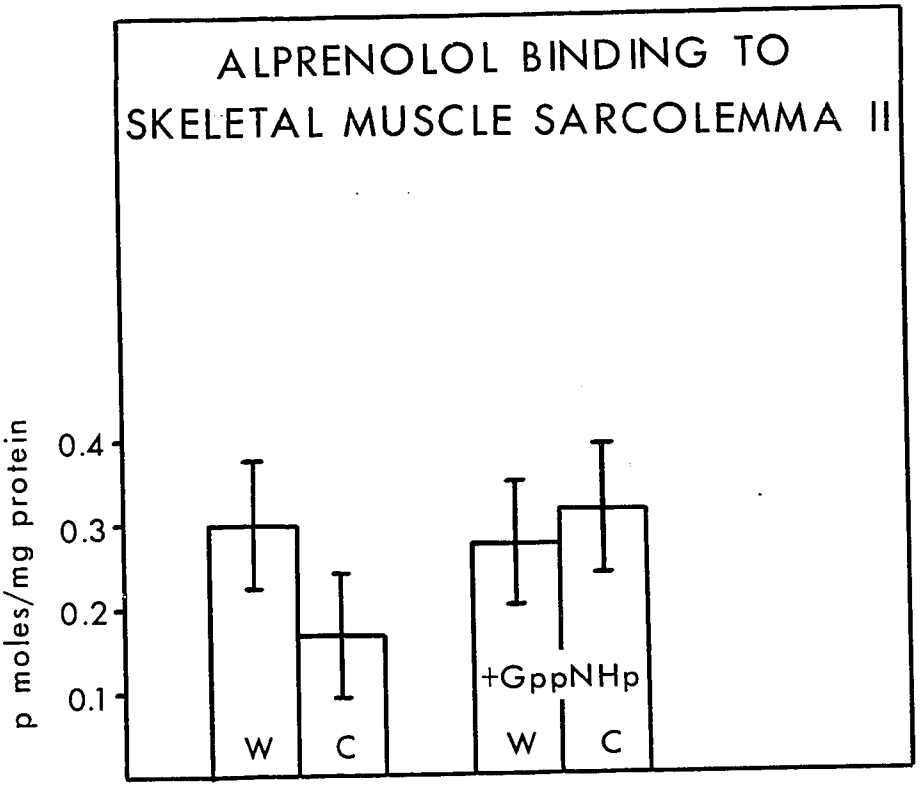
The figure compares (-)[^{3}H] dihydroalprenolol binding to sarcolemma from warm- and cold-acclimated rats, with and without GppNHp (0.12 mM) in the incubation medium. The bars are the means ± SEM of specific binding, i.e. the difference between total binding and binding in presence of propranolol (100 µM). Values are the following (pmoles/mg protein) (n = 3):

	W		C
without GppNHp	0.299 ± 0.115	NS	0.168 ± 0.072
		NS	NS
with GppNHp	0.274 ± 0.072	NS	0.314 ± 0.074

The following table indicates the values obtained for binding of (-)[^{3}H] dihydroalprenolol in each experiment for the warm- and the cold-acclimated rats with and without GppNHp.

Experiment	WA	CA	CA + GppNHp	WA + GppNHp
1	0.528	0.306	0.450	0.394
2	0.192	0.132	0.194	0.284
3	0.176	0.066	0.299	0.145

Abbreviations: W: warm-acclimated
C: cold-acclimated
GppNHp: 5'-guanylyl imidodiphosphate


ALPRENOLOL BINDING TO
SKELETAL MUSCLE SARCOLEMMA II
p moles/mg protein
0.4
0.3
0.2
0.1
W
C
+GppNHp
W
C

parations I & II respectively). These decreases in the two preparations are not significant due to variations in binding values from one experiment to the other and the small number of experiments. However, when the data obtained for the two preparations are pooled, a paired t-test shows that the decrease in (-)[^{3}H]DHA binding is significant ($d = 0.188 \pm 0.065$, $P < 0.025$). The decrease is no longer observed when GppNHp is present in the incubation medium. The binding data obtained with GppNHp in the CA rat preparations equal those obtained in the WA preparations with or without GppNHp. The significance of this observation is also assessed by treatment of the pooled data of preparation I and II by the paired t-test: a significant difference between CA and CA + GppNHp is observed: ($d = 0.159 \pm 0.050$; $P < 0.020$).

As suggested by the concentrations of catecholamines in urine (see Fig. 3), the level of catecholamines circulating in the CA rat is higher. Thus, one can expect a desensitization of adrenergic receptors to occur. This is probably what is happening in the CA rat skeletal muscle. This desensitization is reflected by a decreased number of (-)[^{3}H]DHA binding sites. Binding comes back to control values when GppNHp is present in the incubation medium: GppNHp resensitizes the desensitized receptors. The lack of resensitization by GppNHp in the WA rat preparations probably reflects the absence of desensitization of the β-adrenergic receptors. Desensitization by catecholamines and resensitization by GppNHp has been observed in frog erythrocyte membranes (174) (see section II.3.B.iv.).

Are these effects related to nonshivering thermogenesis? It appears, at first, that desensitization of β-

adrenergic receptors in CA rat sarcolemma is the normal response to high levels of circulating catecholamines, however, resensitization, if it occurs in vivo as well as in vitro (GTP might be the resensitizing agent in vivo) could allow the cell to respond to catecholamines with the maximum intensity. The concentration of the resensitizing agent would thus regulate the response to catecholamine action.

Lefkowitz and coworkers have reported a parallel decrease in isoproterenol-stimulated adenylate cyclase with desensitization of the β-adrenergic receptors (172,174). No similar phenomenon was observed because catecholamine stimulation of adenylate cyclase is not detectable in preparations I & II.

Little work has been done with (-)[^{3}H]DHA binding in relation with cold acclimation. In brown adipose tissue from CA rats a 41% reduction in (-)[^{3}H]DHA binding sites was reported by Bukowiecki and coworkers (25). However, no similar decrease was observed in skeletal muscle by the same group (228). The discrepancy between these results and the results reported in this thesis might come from the fact that Bukowiecki and coworkers used rats which had been at least six weeks in the cold (Bukowiecki, personal communication). As shown in Fig. 3, the level of catecholamines circulating in a CA rat, as estimated by urinary excretion, progressively decreases with the time spent in the cold; it is thus possible that a desensitization occurs when the level of catecholamines is high, and is followed by a progressive resensitization due to the decrease of catecholamine level.

Hence, after a certain number of weeks in the cold, desensitization in the CA rat would not be detectable.

In conclusion, this phenomenon of β-adrenergic receptor desensitization-resensitization does not seem to be related to the enhanced response to noradrenaline seen in CA rats, however, a time-course study would be useful to follow the phenomenon during acclimation to cold and for several weeks when the rat is fully acclimated to cold.

PART 8 : LIPID COMPOSITION

A. Purpose of the experiment.

It is known that Na,K-ATPase activity is highly dependent on the composition of its lipid environment (see section II.3.A.i). It appeared then interesting to check whether the lipid composition of sarcolemma was different in the CA rat. A difference in the lipid composition could explain the increase observed in Na,K-ATPase activity.

In addition, this study would permit the characterization of the isolated plasma membranes.

B. Description of the experiment.

3 WA rats (434 ± 24 g) and 3 CA rats (367 ± 13 g) were used for preparation I, 2 WA rats (448 ± 10 g) and 2 CA rats (320 ± 20 g) for preparation II. The phospholipid and cholesterol contents were measured and the fatty acids analysed as described in methods (III.2.I).

C. Results and discussion.

C(i) Results

Tables III & IV summarize the data obtained for phospholipid composition with technique I and II respectively. The results are given in µmoles/mg protein as well as percentages, and are similar in both preparations and for the WA and the CA rats. The highest value is obtained for phosphatidylcholine (PC), followed by phosphatidylethanolamine (PE); phosphatidylserine (PS)+ phosphatidylinositol (PI) and sphingomyelin (Sph) represent a small proportion of the total phospholipids.

Table V includes values for free cholesterol and cholesterol esters (µg/mg protein). Total cholesterol is also indicated in µmoles/mg protein to allow calculation of the molar ratio of cholesterol to phospholipid (also shown on Table V). Cholesterol concentrations are similar in both preparations and for the WA and the CA rats. Free cholesterol and cholesterol esters represent respectively about 90% and 10% of total cholesterol. Cholesterol to phospholipid ratio is similar for the WA and the CA rats although higher in preparation I.

Finally, table VI collects the percentages of the different fatty acids contained in each of the four phospholipid fractions extracted from both preparations, namely: PE, PC, PS + PI and Sph. The degree of unsaturation for each phospholipid is also included as well as the percentage change in unsaturation from WA to CA animals.

As far as membrane characterization is concerned the percentage of each fatty acid in both preparations (I & II)

Table III. Phospholipid composition of skeletal muscle sarcolemma isolated with technique I (warm- and cold-acclimated rats).

	WA		CA	
	µmoles/mg protein	%	µmoles/mg protein	%
PE	0.071 ± 0.003	27 ± 1	0.071 ± 0.023	29 ± 9
PC	0.174 ± 0.009	67 ± 4	0.148 ± 0.032	60 ± 13
PS + PI	0.002 ± 0.001	0.5 ± 0	0.019 ± 0.016	8 ± 6
Sph	0.013 ± 0.003	5 ± 1	0.010 ± 0.007	4 ± 3
total phospholipid	0.260		0.248	

Results are given as mean ± SEM of 3 experiments. The differences between warm- and cold-acclimated rats for each phospholipid are not significant.

Abbreviations: PE : phosphatidylethanolamine
PC : phosphatidylcholine
PS + PI : phosphatidylserine + phosphatidylinositol
Sph : sphingomyelin

Table IV. Phospholipid composition of skeletal muscle sarcolemma isolated with technique II (warm- and cold-acclimated rats).

	WA		CA	
	µmoles/mg protein	%	µmoles/mg protein	%
PE	0.077 ± 0.010	20 ± 0	0.094 ± 0.007	21 ± 2
PC	0.281 ± 0.036	71 ± 9	0.319 ± 0.003	72 ± 1
PS + PI	0.011 ± 0.011	3 ± 3	0.007 ± 0	2 ± 0
Sph	0.024 ± 0.008	6 ± 2	0.021 ± 0.015	5 ± 3
total phospholipid	0.392		0.441	

Results are given as mean ± SEM of 2 experiments. The differences between warm- and cold-acclimated rats for each phospholipid are not significant.

Abbreviations: PE : phosphatidylethanolamine
PC : phosphatidylcholine
PS + PI : phosphatidylserine + phosphatidylinositol
Sph: sphingomyelin

Table V. Cholesterol content and cholesterol to phospholipid molar ratio of skeletal muscle sarcolemma isolated with techniques I and II (warm- and cold-acclimated rats)

		Free cholesterol µg/mg protein	Cholesterol esters µg/mg protein	Total cholesterol µmoles/mg protein	$\frac{\text{cholesterol}}{\text{phospholipid}}$
technique I	W	32.6 ± 1.0	3.8 ± 2.4	0.091	0.35
	C	26.4 ± 3.9	3.25 ± 1.15	0.074	0.30
technique II	W	28.0 ± 1.5	1.3 ± 0.8	0.075	0.19
	C	32.8 ± 0.4	2.7 ± 2.0	0.089	0.20

Values are means ± SEM (µg/mg protein) of 2 experiments. Total cholesterol is given in µmoles/mg protein. Differences are not significant.

The following molecular weights have been taken for free cholesterol and cholesterol esters.

Free cholesterol : 386
Cholesterol ester : 627
(esterified by palmitic acid)

Abbreviations: W : warm-acclimated
C : cold-acclimated

Table VI. Fatty acid composition and degree of unsaturation of phospholipids from skeletal muscle sarcolemma isolated with techniques I and II (warm- and cold-acclimated rats).

	Phosphatidylethanolamine				Phosphatidylcholine				Phosphatidyl-serine -inositol				Sphingomyelin			
	I		II		I		II		I		II		I		II	
	W	C	W	C	W	C	W	C	W	C	W	C	W	C	W	C
14:0	<0.1	<0.1	0.1	0.2	0.1	<0.1	0.2	<0.1	1.2	0.3	0.2	0.2	0.1	0.2	0.3	0.3
16:0	3.5	4.2	6.0	7.0	38.6	34.1	38.3	40.6	8.0	10.4	4.3	5.4	11.2	11.5	21.4	25.4
16:1	0.4	0.3	0.9	0.9	0.8	0.6	1.2	0.7	5.5	0.5	3.4	2.2	3.5	3.4	4.8	2.6
18:0	23.5	27.3	16.0	18.9	10.0	13.4	8.1	8.9	21.9	35.7	42.2	36.4	50.7	45.7	22.0	31.1
18:1	3.0	2.5	6.2	7.1	6.9	6.5	7.5	6.7	18.7	7.4	8.1	6.5	6.1	6.4	11.6	8.8
18:2	7.9	6.5	4.6	5.7	22.8	21.6	22.5	21.1	6.3	6.1	2.2	3.5	1.0	1.6	9.7	10.9
18:3			0.6	0.5					6.0		2.5	1.7	3.2	4.1	4.8	1.4
20:4	15.6	12.0	9.0	8.7	13.7	13.9	14.0	13.1	10.0	20.7	21.9	26.4	3.0	5.2	4.2	2.8
22:6	33.2	33.7	40.9	37.1	5.6	9.7	6.4	8.1	3.5	17.9	8.4	13.5	5.2	4.8	4.5	5.0
Degree of unsaturation double bond per mole	2.80 ±0.0	2.66 ±0.17	2.97 ±0.18	2.78 ±0.08	1.41 ±0.05	1.63 ±0.03	1.48 ±0.03	1.50 ±0.01	1.14 ±0.08	2.10 ±0.05	1.61 ±0.23	2.07 ±0.17	0.64 ±0.11	0.68 ±0.21	0.89 ±0.03	0.75 ±0.02
% change		-5 ±6		-6.5 ±8		+15.5 ±2.5		+1.5 ±1.5		+84 ±16		+28.5 ±8		+3 ±16		-16.5 ±0.5

In this table are given the percentages of each fatty acid present in each phospholipid extracted from a given preparation as indicated : from left to right, the odd columns give the results (means of 2 experiments) of preparation I for warm- and cold-acclimated rats, whereas the even columns give the results (means of 2 experiments) of preparation II. Degree of unsaturation is the sum of each percentage multiplied by the respective number of double bonds and divided by 100. The last line indicates the percentage change in fatty acid unsaturation (± SEM) between the warm- and cold-acclimated rats.

Abbreviations: fatty acids are expressed by their number of carbon atoms and number of double bonds. e.g. linoleate = 18:2 . palmitate = 16:0 . W: warm-acclimated . C: cold-acclimated.

is similar for PE and PC but different for PS + PI and Sph. However, the degree of unsaturation is similar in all the fractions studied for both preparations (I & II), the higher degree of unsaturation being observed for PE because of its high content of 22:6 fatty acid.

As far as cold acclimation is concerned, an increase in the fatty acid degree of unsaturation of the PS + PI fraction is observed in both preparations (I & II).

A large percentage of stearic acid (18:0) appears in PE, PS + PI and Sph fractions. PC contains a high proportion of palmitic acid (16:0). Polyunsaturated fatty acids predominate in PE (20:4 and 22:6), PC (18:2) and PS + PI (20:4).

In Fig. 31, results from tables III, IV and VI are summarized and compared. The percentage of each phospholilid is given at the top of the figure for the two categories of rats and both preparations. The diagram under these numbers is an expression of the degree of unsaturation of each phospholipid. This degree is fixed at 100% (horizontal line) for the WA rat; the bars represent the degree of unsaturation of fatty acids from the CA animal as percentages of the WA animal.

C(ii). Discussion.

The amount of phospholipid of each fraction is lower than reported in the literature; the proportion of PC is higher and the proportion of Sph and PS + PI lower (47, 53).

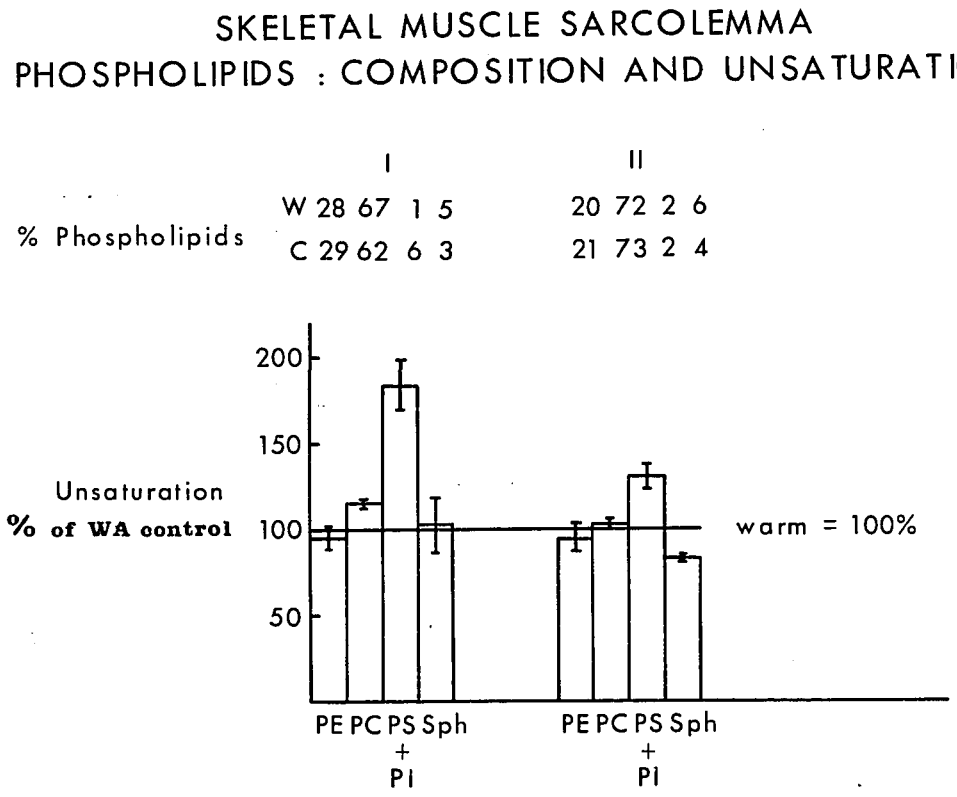


Fig. 31. Phospholipid composition and degree of unsaturation of skeletal muscle sarcolemma isolated with techniques I and II (warm- and cold-acclimated rats).

Results for preparations I and II are placed respectively on the left and the right of the figure. The percentages of the various phospholipids indicated in abscissa are presented at the top of the figure. The bars represent the percentage changes in unsaturation of phospholipid fatty acids from the cold-acclimated rat sarcolemma, the degree of unsaturation of the warm-acclimated rat preparation being fixed at 100%.

Abbreviations:
PE : phosphatidylethanolamine
PC : phosphatidylcholine
PS + PI : phosphatidylserine + phosphatidylinositol
W : warm-acclimated
C : cold-acclimated

This figure represents the results combined from tables III, IV and VI.

Values for cholesterol and cholesterol to phospholipid ratio are in the range found in literature (47,53,127).

Because of the small contribution of PS + PI fraction to the total phospholipid composition (2 - 6%) (see tables III & IV and Fig. 31), it is unlikely that the increase in unsaturation of the fatty acids of this fraction could be responsible for any change in fluidity of the membrane. However, it is possible that the change in Na,K-ATPase reported above (see section IV.5.) might be a consequence of this increase, if one considers the importance of lipids in Na,K-ATPase activity (see section II.3.A.i.).

PART 9 : POLYPEPTIDE COMPOSITION.

A. Purpose of the experiment.

As in the preceding sections, the purpose of this experiment was dual: polypeptide composition was studied first to characterize the plasma membranes, secondly to find out whether the changes described above in the CA rat (Na,K-ATPase, β-adrenergic receptors) would be accompanied by a different protein composition.

B. Description of the experiment.

For polypeptide composition of preparation I, 5 WA rats (460 ± 16 g) and 4 CA rats (386 ± 10 g) were used. 5 WA (434 ± 30 g) and 5 CA rats (364 ± 19 g) served for preparation II. Plasma membranes were analysed for polypeptides as described in methods (III.2.J.).

C. Results and discussion.

The results are presented in Fig. 32 & 33 for preparation I from the WA and CA rats respectively, whereas Fig. 34 & 35 illustrate the polypeptide patterns of preparation II from the WA and CA rats respectively.

A total of 20 different distinct polypeptides are present in preparation I ranging from 23 000 to 255 000. In preparation II, 19 polypeptides are visible, ranging from 15 500 to 256 000. 13 polypeptides are common to both preparations and are numbered from 1 to 13. The 7 polypeptides left in preparation I and which are present only in this preparation are designated by letters A to G. In a similar manner, the 6 polypeptides left in preparation II and specific to this preparation are designated by letters H to M. A summary of the results reported in Fig. 32 to 35 is given in table VII. The peaks are classified according to their numbering. Possible identification of the peaks is also given with the reference in the literature. The table also indicates the peaks which, although showing different molecular weights, could correspond to the same polypeptide in preparation I and II.

C(i). Plasma membrane characterization.

The characterization will be discussed at three levels:

- comparison between preparations I and II.
- comparison of preparations I and II with data reported in the literature.
- identification of the various polypeptides.

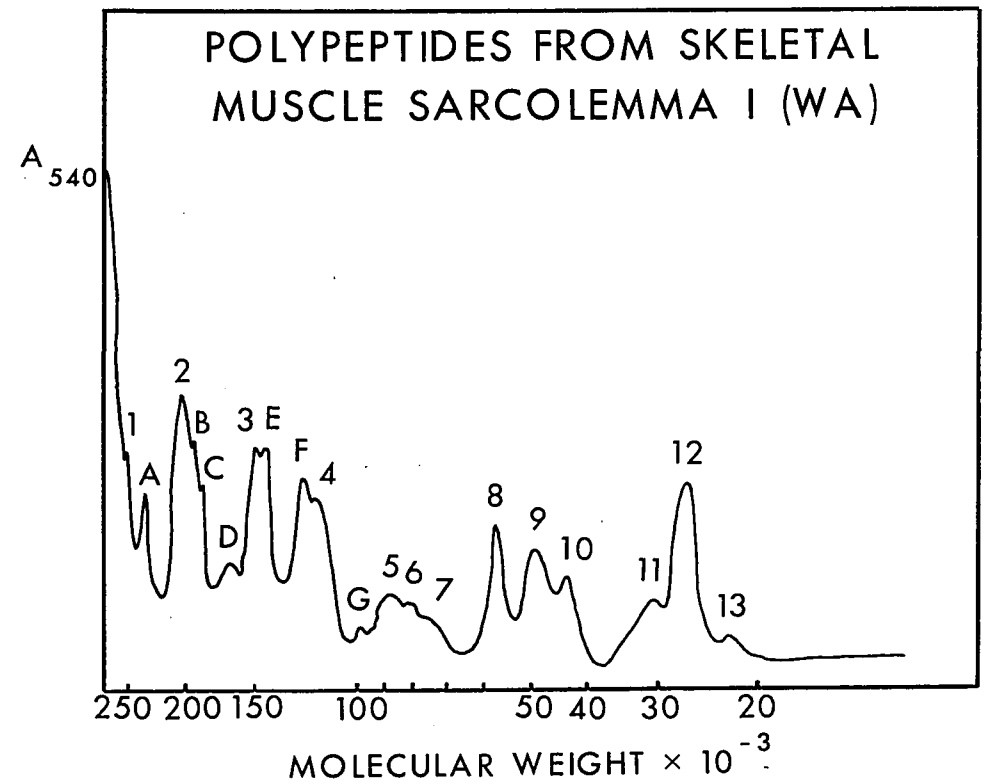


Fig. 32. Polypeptide pattern of skeletal muscle sarcolemma isolated with technique I (warm-acclimated rat).

The graph shows a typical scan at 540 nm of the warm-acclimated (WA) rat preparation after SDS-polyacrylamide gel electrophoresis.

The molecular weights for the different peaks are the following (means of 5 different experiments):

1	251 000	G	99 500
A	230 000	5	89 000
2	197 000	6	85 000
B	192 000	7	77 500
C	183 000	8	58 500
D	163 000	9	50 500
3	150 000	10	44 000
E	143 000	11	32 500
F	125 000	12	27 500
4	119 000	13	23 000

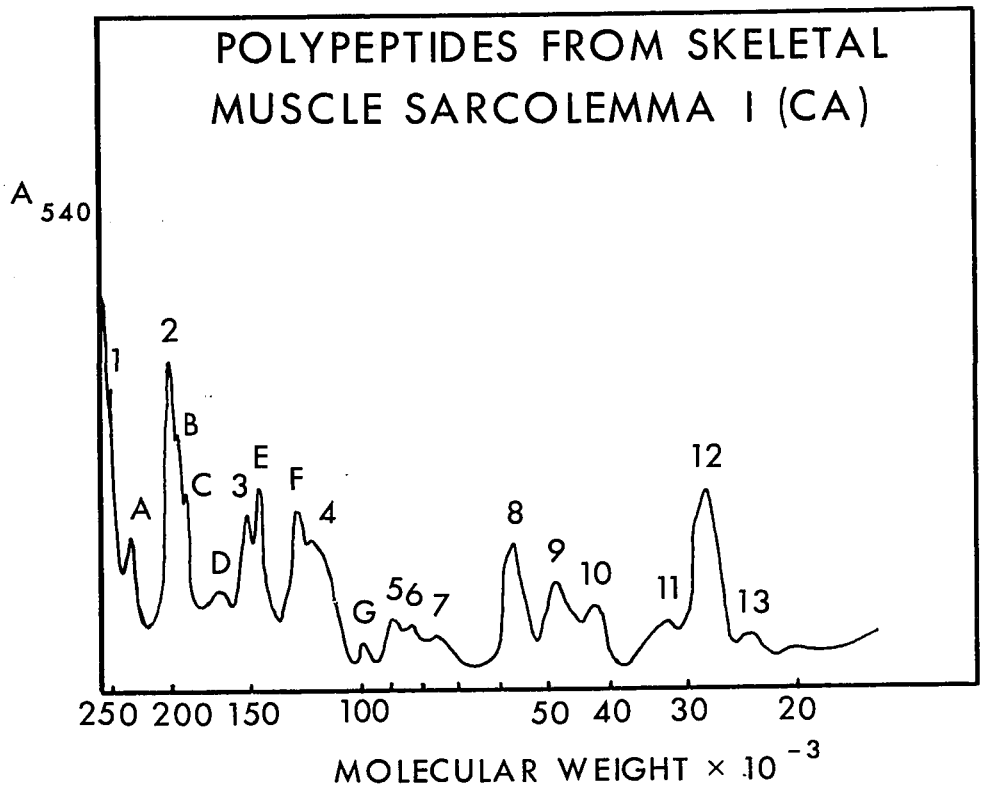


Fig. 33. Polypeptide pattern of skeletal muscle sarcolemma isolated with technique I (cold-acclimated rat).

The graph shows a typical scan at 540 nm of the cold-acclimated (CA) rat preparation after SDS-polyacrylamide gel electrophoresis.

The molecular weights for the different peaks are the following (means of 4 different experiments):

1	255 000	G	98 500
A	230 000	5	88 500
2	202 000	6	83 000
B	194 000	7	76 000
C	188 000	8	57 500
D	166 000	9	50 000
3	150 000	10	43 000
E	144 000	11	32 500
F	125 000	12	27 500
4	118 000	13	24 000

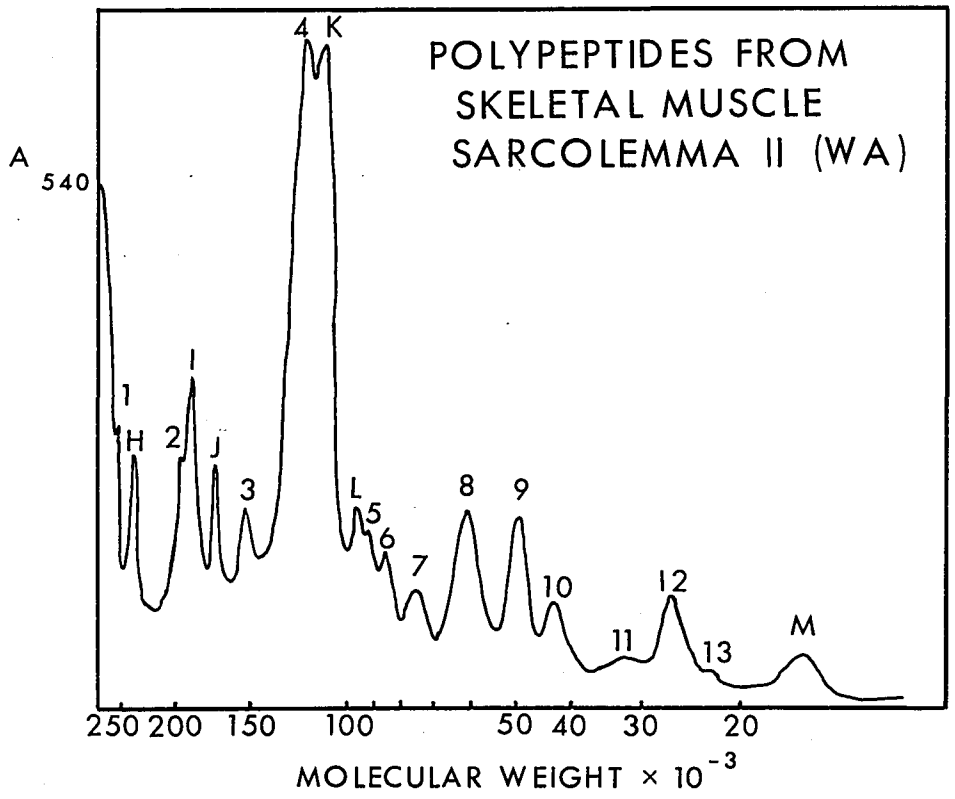


Fig. 34. Polypeptide pattern of skeletal muscle sarcolemma isolated with technique II (warm-acclimated rat).

The graph shows a typical scan at 540 nm of the warm-acclimated (WA) rat preparation after SDS-polyacrylamide gel electrophoresis.

The molecular weights for the different peaks are the following (means of 5 different experiments).

1	256 000	6	84 500
H	238 000	7	75 000
2	196 000	8	60 500
I	187 000	9	49 000
J	171 000	10	43 000
3	150 000	11	31 500
4	118 000	12	26 500
K	110 000	13	23 000
L	96 000	M	15 500
5	90 000		

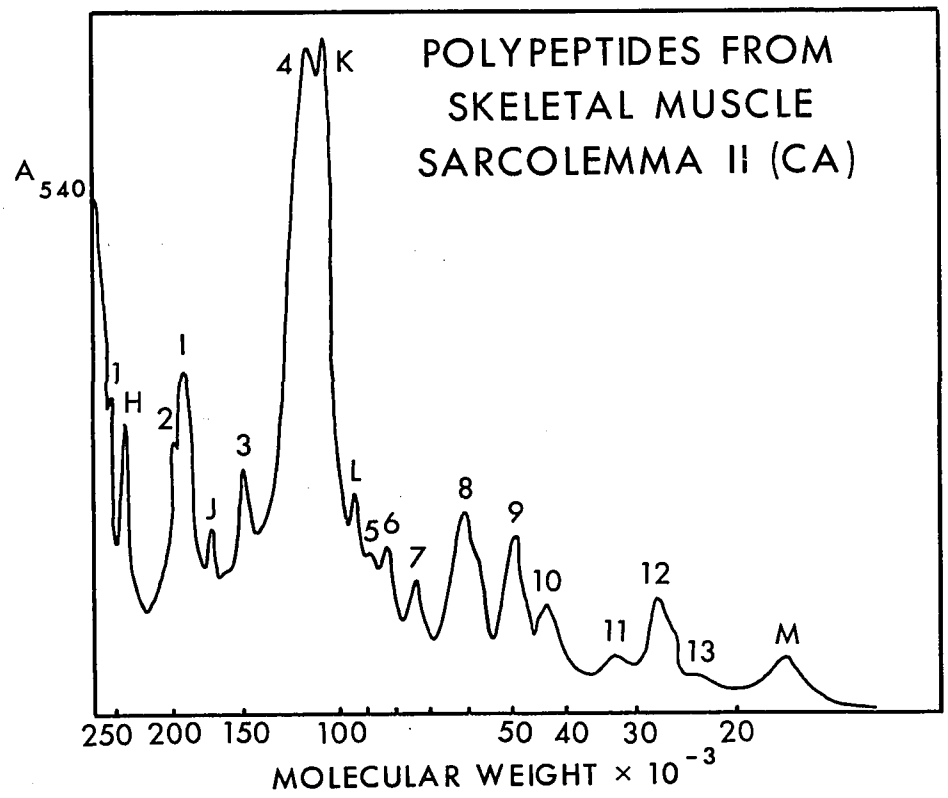


Fig. 35. Polypeptide pattern of skeletal muscle sarcolemma isolated with technique II (cold-acclimated rat).

The graph shows a typical scan at 540 nm of the cold-acclimated (CA) rat preparation after SDS-polyacrylamide gel electrophoresis.

The molecular weights for the different peaks are the following (means of 5 different experiments):

1	256 000	6	83 000
H	240 000	7	73 000
2	200 000	8	58 500
I	191 000	9	48 500
J	170 000	10	42 000
3	148 000	11	30 500
4	117 000	12	26 000
K	109 000	13	23 000
L	94 500	M	15 500
5	87 000		

Table VII. Polypeptides of skeletal muscle sarcolemma isolated with techniques I and II (warm- and cold-acclimated rats).

Peak	Common	I W	I C	II W	II C
1		251 000	255 000	256 000	256 000
2		197 000	202 000	196 000	200 000
3	β-adrenergic receptor (32,61)?	150 000	150 000	150 000	148 000
4		119 000	118 000	118 000	117 000
5	Na,K-ATPase: large	89 000	88 500	90 000	87 000
6	subunit (46,121)?	85 000	83 000	84 500	83 000
7		77 500	76 000	75 000	73 000
8		58 500	57 500	60 500	58 500
9	Na,K-ATPase: small subunit (46,121)?	50 500	50 000	49 000	48 500
10	Ca^{++}-binding protein (229)?	44 000	43 000	43 000	42 000
11	β-adrenergic receptor (7)?	32 500	32 500	31 500	30 500
12		27 500	27 500	26 500	26 000
13		23 000	24 000	23 000	23 000
	Possibly different				
H				238 000	240 000
A		230 000	230 000		
B		192 000	194 000		
C		183 000	188 000		
I				187 000	191 000
J				171 000	170 000
D		163 000	166 000		
E	β-adrenergic receptor (32,61)?	143 000	144 000		
F		125 000	125 000		
K				110 000	109 000
G	Sarcoplasmic reticulum (5)?	99 500	98 500		
L	Sarcoplasmic reticulum (5)?			96 000	94 500
M				15 500	15 500

This tablescompares the polypeptides obtained with preparations I and II, and indicates their possible identifications according to the literature. In the upper part of the table are listed the peaks common to both preparations (peaks 1 to 13), whereas the lower part shows the peaks found in one preparation but not in the other. The arrows indicate the peaks which might be common to both preparations although their molecular weights are slightly different.

Abbreviations:
W : warm-acclimated
C : cold-acclimated
I : technique I
II : technique II

Preparation I differs from preparation II for the large molecular weight polypeptides (> 90 000). Only 4 polypeptides (peaks 1 to 4) are common to both preparations in the region. However, 9 polypeptides are found in both preparations in the range 20 000 to 90 000. Actually, all the peaks present in this region in preparation I are also found in preparation II, although with a different magnitude. The profile of the scan is very similar for polypeptides 5 to 13 in both preparations. The discrepancy observed in the 90 000 to 250 000 region (especially the increase in peak 4 and the presence of a huge amount of peak K in preparation II) is not surprising considering the total lack of similarity between the isolation techniques used and also between the results obtained (tubes versus vesicles). A difference in polypeptide pattern is also reported by Boegman who used techniques I and II. Although his molecular weight values are not available, it is in the high molecular weight region that the inconsistency is observed (22).

As already mentioned (II.4.B.ii.), very little information is available in the literature on sarcolemmal proteins. In addition, the results reported by the different authors (5,22, 162,229) are not consistent from one author to the other. Vandenburgh (229) reports 3 major bands with molecular weights of 170 000, 140 000 and 44 000. A minor 44 000 peak is common to preparations I and II but no 140 000 or 170 000 polypeptide is present in both preparations: a 143 000 peak

(E) visible in preparation I whereas a 171 000 peak (J) is present only in preparation II. A major band around 44 000 appears also in Madeira & Antunes-Madeira (162) preparation while a 46 000 peak is present in the pattern obtained by Andrew & Appel (5).

According to Vandenburgh et al., (229) the 44 000 polypeptide could correspond to a calcium-binding protein present in large quantities in the sarcoplasmic reticulum. In such case, contamination of preparations I and II by sarcoplasmic reticulum should be minor (small peak) and similar in both preparations (same magnitude). Andrew and Appel (5) report the existence of a large peak at 93 000 in a fraction corresponding to sarcoplasmic reticulum. This band is also present in the plasma membrane enriched fraction but to a lesser extent. The absence of such a peak in preparations I and II is a confirmation of the lack of contamination of these preparations by sarcoplasmic reticulum. The difference observed in peak 4 between preparations I and II might be explained either by a loss of the corresponding polypeptide in preparation I due to the strong salt concentrations used in technique I or by contamination of preparation II by other subcellular membrane fractions. A similar interpretation might be given to explain the presence of peak K in preparation II.

Na,K-ATPase has been reported to appear as two peaks on SDS gels, ranging from 84 000 to 100 000 and from 47 000 to 56 000 (46, 121). Preparations I & II display 3 peaks in

the 84 000 - 100 000 region, two of them (5 & 6) being common to both. One of them could correspond to the Na,K-ATPase highest molecular weight polypeptide, however the peaks in this area are not well enough defined to draw any definitive conclusion. In the 47 000 to 56 000 region, only peak 9 is visible, corresponding to 48 500 - 50 500 and could be the smaller molecular weight component of Na,K-ATPase.

β-Adrenergic receptors appear to have a molecular weight ranging from 130 000 to 150 000 (32,61). Two bands in the range 37 000 - 41 000 have also been reported by gel electrophoresis (7). Peak 3 could be candidate as well as peak E in preparation I. Peaks 10 or 11 could correspond to the smaller molecular weight polypeptide reported in the literature (7).

In conclusion, characterization of plasma membranes by protein composition appears difficult for two reasons:

- Proteins being very sensitive to plasma membrane isolation conditions, the results obtained vary with the isolation technique used.
- Pure plasma membrane fraction is very difficult to obtain, thus any other subcellular membrane fraction will contribute with its own specific proteins to the final protein pattern.

C(ii). Polypeptide composition and cold acclimation.

Do the changes in the CA rat reported in this thesis appear in the polypeptide patterns? The scans from the WA

rat and the CA rat look very similar in both preparations (I & II).

The decrease in (-)[^{3}H]DHA binding to the CA rat preparation (see section IV.H.) has been interpreted as a desensitization of the β-adrenergic receptor sites. These sites are inactivated but still present, since incubation with GppNHp leads to resensitization. Thus, it is unlikely that desensitization could appear as a decrease in the polypeptide(s) presumably corresponding to the β-adrenergic receptor site. Indeed, the magnitude of the 150 000 peak (peak 3) is the same in preparations from the WA and the CA rats. Peak E present in preparation I has also the same magnitude in the WA and the CA rats.

As mentioned above, Na,K-ATPase appears as two peaks: a large one ranging from 84 000 to 100 000 (presumably peak 5 or 6) and a smaller one with a molecular weight between 47 000 & 56 000 (presumably peak 9). As indicated by the polypeptide patterns (Fig. 32 to 35), the increase in Na,K-ATPase activity observed in the CA rat sarcolemmal preparation (see section IV.5.C.ii), is apparently not accompanied by an increase in the number of pumping sites. However a definite conclusion cannot be drawn, for the following reasons:

- the Na,K-ATPase has not been identified for certain in the protein patterns.
- the increase, if present, might be too low to be visible as a change in the amount of protein.

This increase can still be explained by a change in the affinity of the enzyme for the substrate (ATP) or as a consequence of the observed change in lipid unsaturation as already considered (see section IV.8.C.).

CHAPTER V: CONCLUSIONS

The purpose of the study reported in this thesis was to identify the biochemical mechanism responsible for the enhanced calorigenic response to noradrenaline by the cold-acclimated rat, i.e. for nonshivering thermogenesis. It was assumed that skeletal muscle was a major site of this response and that an altered capacity of the muscle to respond to noradrenaline might be associated with changes in its plasma membrane. The approach used was to compare the sarcolemma from the cold-acclimated rat with the sarcolemma from the control (warm-acclimated) rat.

For this purpose, sarcolemma was isolated by two different techniques and characterized. As far as isolation is concerned, it appears that both techniques (extraction by high salt concentrations and isolation on a sucrose density gradient) can be used to study the properties of sarcolemma. However, the two preparations used differ considerably in their properties. Thus, preparation II has higher Na,K-ATPase, Mg-ATPase and adenylate cyclase activities but lower 5'-nucleotidase activity. The Na,K-ATPase of preparation II is not inhibited by ouabain whereas that of preparation I is inhibited. These results might be explained by the vesicles isolated with technique II being inside-out. This would increase the accessibility of the ATPases and adenylate cyclase to their substrate (ATP) and reduce the accessibility of Na,K-ATPase to ouabain [binding sites are known to be on

the outer surface (93)] and of 5'-nucleotidase to AMP [enzyme known to be on the outer surface (239)]. This hypothesis could be verified by use of radioactive ouabain: binding of [^{3}H]-ouabain should be low in preparation II.

As far as cold acclimation is concerned, the major findings are the following:

- Na,K-ATPase activity is increased in the skeletal muscle sarcolemma of the cold-acclimated rat. However, this increase is not accompanied by a detectable change in polypeptide composition. It might be a consequence of the increased fatty acid unsaturation observed in the phosphatidylserine + phosphotidylinositol fraction of membrane phospholipids. No effect of noradrenaline on Na,K-ATPase activity is observed.

- β-Adrenergic receptor concentration is reduced in the skeletal muscle sarcolemma of the cold-acclimated rat. This reduction is not reflected in the polypeptide composition. It probably corresponds to a desensitization rather than a loss of receptor sites, as indicated by the resensitization observed under action of GppNHp.

- No consistent changes are obtained in the activities of Mg-ATPase, 5'-nucleotidase, adenylate cyclase (basal, fluoride-, noradrenaline-, GppNHp-, GppNHp + noradrenaline-stimulated) or in lipid composition and polypeptide composition.

The changes observed at the level of the skeletal muscle plasma membrane of cold-acclimated rats do not allow any satisfactory explanation of the enhanced calorigenic response of catecholamines. The reduction in β-adrenergic receptors and the lack of stimulation of adenylate cyclase by noradrenaline would argue against any change in this system being responsible for an enhanced response. A similar reduction in β-adrenergic receptors occurs in brown adipose tissue of cold-acclimated rats (25), yet there is good evidence that the capacity of this tissue to respond calorigenically to catecholamines is increased in the cold-acclimated state (59).

An increase in Na,K-ATPase activity would be in keeping with an increased operation of this enzyme in the intact cold-acclimated animal. However, no direct stimulatory effect of noradrenaline or of cyclic AMP on this enzyme could be detected and the increased operation, if it occurs, must be in response to some other mediator, possibly increased Na^+ levels brought about by the increased permeability to ions known to be induced by catecholamines in skeletal muscle (198). The increase in Na,K-ATPase activity may be related to the increased ouabain-sensitive respiration reported to occur in skeletal muscle of cold-acclimated rats (76).

Some questions remain to be answered:

- Is the increase in Na,K-ATPase activity related to cold acclimation and nonshivering thermogenesis or secondary

to shivering thermogenesis? This question could be answered by a time-course study of the increase in Na,K-ATPase activity during acclimation of the rat to cold.

- Is the increase in Na,K-ATPase activity due to the synthesis of new sodium pump sites or to a change in the affinity of the enzyme for the substrates? A kinetic study of radioactive ouabain binding and a study of the incorporation of radioactive amino acids would be required to answer this question.

- Is the desensitization of β-adrenergic receptors related to the high levels of circulating catecholamines during the initial phase of acclimation to cold? This could also be answered by a time-course study.

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